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THE BIOCHEMICAL EFFECTS OF INJECTIONS OF SEX HORMONES INTO CASTRATED RATS

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A STUDY of the biochemical effects of injections of diethylstilbestrol and testosterone propionate into intact rats has previously been made (Buchwald and Hudson, 1944). The calcium, phosphorus, and acid and alkaline phosphatase activity of the blood serum and the alkaline phosphatase activity of the bone were determined to discover any possible metabolic changes resulting from an excess of either the male or female sex hormone. The importance of these investigations was discussed in view of the clinical changes resulting from the injection of diethylstilbestrol and/or castration in the human with carcinoma of the prostate. It was thought that the changes following hormone injections might be magnified if the amount of the natural hormone were reduced. Accordingly, we have extended our observations to a series of castrated male and female rats treated experimentally in the same manner as the intact rats.

EXPERIMENTAL

Wistar albino rats 110–115 days old were castrated under ether anaesthesia. Injections were started when these animals were 145–155 days old. The experimental procedure and analytical methods were the same as previously described.

DISCUSSION

Table 1 shows the results of injecting 0.1 mg. of diethylstilbestrol daily into castrated male rats. Table 2 shows the results of injecting

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TABLE 1. EFFECTS OF INJECTING DIETHYLSTILBESTROL INTO CASTRATED MALE RATS

	1 Noninjected	2 Oil	3 Diethylstilbestrol	4	5
Number of rats	12	12	12		
Weight difference, gm.	5 ±3.60	6 ±0.99	−37 ±2.71	0.27	14.90
Femurs, fresh weights, gm.					
1.	0.83 ±0.015	0.83 ±0.010	0.86 ±0.10	0.00	2.12
2.	0.84 ±0.016	0.83 ±0.010	0.85 ±0.009	0.53	1.49
Femur, weight of ash, mg.	368.6 ±6.97[1]	371.2 ± 4.84	397.4 ±4.64	0.31	3.91
Serum					
Calcium, mg. %	10.92 ±0.16	10.75 ±0.17	10.57 ±0.17	0.72	0.75
Phosphorus, mg. %	7.02 ±0.21	6.33 ±0.28	7.10 ±0.25	1.98	2.04
Phosphatase, units per 100 cc.					
Acid	8.5 ±0.29	8.4 ±0.44	4.8 ±0.50[1]	0.19	5.41
Alkaline	89.9 ±4.78	85.2 ±5.28	80.0 ±2.77	0.66	0.87
Bone					
Alkaline phosphatase, units per gm.	7.9 ±0.27	7.7 ±0.49	7.2 ±0.49	1.25	2.02

[1] 11 rats

TABLE 2. EFFECTS OF INJECTING TESTOSTERONE PROPIONATE INTO CASTRATED FEMALE RATS

	1 Noninjected	2 Oil	3 Testosterone propionate	4	5
Number of rats	12	12	12		
Weight difference, gm.	0 ±3.70	4 ±1.32	23 ±1.74	1.02	8.70
Femurs, fresh weight, gm.					
1.	0.64 ±0.007	0.62 ±0.008	0.65 ±0.009	1.88	2.49
2.	0.62 ±0.007	0.61 ±0.008	0.64 ±0.009	0.94	2.65
Femur, weight of ash, mg.	284.2 ±3.50[1]	281.9 ±4.27	290.4 ±4.18	0.42	1.42
Tubes, weight, gm.	0.15 ±0.010	0.12 ±0.006	0.30 ±0.17	2.57	9.98
Serum					
Calcium, mg. %	10.48 ±0.18[3]	10.83 ±0.17	10.83 ±0.17[2]	1.43	0.00
Phosphorus, mg. %	6.31 ±0.21[3]	6.62 ±0.24	7.10 ±0.26[2]	0.97	1.36
Phosphatase, units per 100 cc.					
Acid	7.6 ±0.45	7.7 ± 0.38[1]	7.5 ±0.33	0.17	0.40
Alkaline	88.3 ±3.20	85.3 ±3.47	84.1 ±2.71	0.64	0.27
Bone					
Alkaline phosphatase, units per gm.	9.2 ±0.81	8.1 ±0.25	9.7 ±0.24	1.30	4.62

[1] 11 rats
[2] 10 rats
[3] 7 rats

0.2 mg. of testosterone propionate daily into castrated female rats. Columns 1, 2, and 3 show the means with their probable errors. Column 4 shows the statistical comparison between the noninjected and oil injected castrated rats and Column 5 the comparison between the oil and hormone injected castrated rats. These values had to be over three to be considered significant. Table 3 gives the mean results

together with their probable errors of the studies on control male and female intact rats receiving no injections. These results include data previously reported on nine male and six female rats with the addition of a sufficient number of animals to make a total of 12 in each group. A comparison of the results on the intact rats with those on the castrated reported in Tables 1 and 2 shows that the outstanding difference is an increase in the acid phosphatase activity of the blood serum of the castrated rats. Of the six groups (oil injected, hormone injected, and noninjected males and females) each group except the noninjected females showed a significant increase in the acid phosphatase activity of the serum. From clinical experiences with castration in human pathological cases this increase in serum acid phosphatase activity is contrary to expected results. Dean, Woodard, and

TABLE 3. NONINJECTED INTACT RATS

	Male	Female
Number of rats	12	12
Weight difference, gm.	19 ±2.79	9 ±1.29
Femurs, fresh weights, gm.		
1.	0.85 ±0.017	0.62 ±0.010
2.	0.85 ±0.018	0.62 ±0.009
Femur, weight of ash, mg.	387.6 ±8.82	294.6 ±4.72
Sex organs, weight, gm.	2.71 ±0.029	0.69 ±0.014
Serum		
Calcium, mg. %	11.04 ±0.145	10.93 ±0.168
Phosphorus	7.03 ±0.223	6.20 ±0.266
Phosphatase, units per 100 cc.		
Acid	6.6 ±0.42	7.0 ±0.44
Alkaline	69.4 ±3.09	64.2 ±2.33
Bone		
Alkaline phosphatase, units per gm.	9.2 ±0.78	8.5 ±0.34

Twombly (1944) considering their patients with carcinoma of the prostate report, "In patients in whom acid phosphatase does not enter the circulation neither castration nor administering stilbestrol causes it to do so." However, in our experience castration of normal male or female rats caused an increase in the serum acid phosphatase activity. The same workers further report that castration decreases the urinary excretion of estrogenic hormones, markedly increases the pituitary gonadotropic hormones, and does not affect the androgenic hormones. If the urinary excretion of these hormones can be taken as an index of their concentration in the organism, the estrogenic hormones in male rats being suppressed by castration, a favorable condition exists for the increased activity of serum acid phosphatase. In the castrated female rats the same condition exists but the source of the enzyme is obscured. If the permeability of the erythrocytes, which contain an acid phosphatase, is increased an increase of the enzyme activity might be observed. In our experience the rat erythrocytes are normally much more fragile than the human, probably accounting for the higher normal level of serum acid phosphatase in the rat than

in the human. On the other hand, instead of an actual increase in the enzyme there is the possibility that removal of the sex organs effects the removal of an inhibitor, producing only an apparent increase of the enzyme.

In general, the effect of injecting sex hormones was the same in the castrated animals as in the intact. The expectation of emphasizing the effects of the injections of sex hormones was not realized in the castrated rats. Hormone injections produced no change in the calcium or phosphorus of the blood serum of either male or female rats. In the castrated male rats receiving diethylstilbestrol injections the alkaline phosphatase activity of the serum was unchanged but the acid

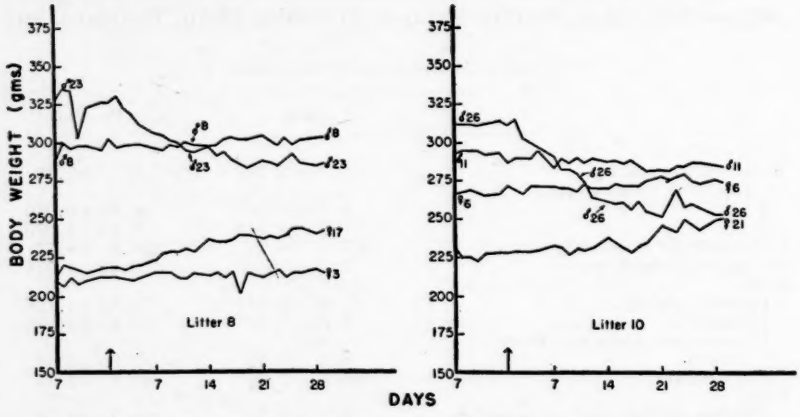

FIG. 1. Daily body weights of castrated rats on metabolism experiment. Females 3 and 6, males 8 and 11 injected with 0.1 cc. of sesame oil. Females 17 and 21 injected with 0.2 mg. of testosterone propionate in 0.1 cc. of sesame oil. Males 23 and 26 injected with 0.1 mg. of diethylstilbestrol in 0.1 cc. of sesame oil. Arrow indicates day injections were started.

phosphatase activity was decreased though not to the level in the intact animals since the acid phosphatase activity was initially higher in the castrated rats than in the intact. In the females atrophy of the uteri occurred following castration but on injection of testosterone propionate the weight of the uteri increased in accordance with the observations of Mazer and Mazer (1940).

In the castrated rats receiving hormone injections the alkaline phosphatase activity of the femurs of the female rats increased but there was no change in the femurs of the male rats. In the intact rats receiving hormones the alkaline phosphatase activity of the femurs of the male rats decreased and there was no change in the femurs of the females. On the basis of these results produced in the males it might be more advantageous to treat patients with metastatic carcinoma of the prostate by both stilbestrol administration and castration rather than stilbestrol alone.

Figure 1 shows the daily body weights of the rats on the metabolism experiment. Table 4 shows the calcium and phosphorus intake and output of these rats. These data demonstrate no effect on either calcium or phosphorus metabolism by diethylstilbestrol and testosterone propionate injections in spite of the fact that the injected hormones were acting without the presence of the natural hormones produced by the sex glands.

Castration increased the level of serum acid phosphatase activity in both male and female rats, an unexpected result in the light of our

TABLE 4. EFFECTS OF THE INJECTION OF SEX HORMONES ON THE CALCIUM AND PHOSPHORUS METABOLISM IN CASTRATED RATS

	Calcium intake mg. Ca	Calcium output mg. Ca	Calcium balance mg. Ca	Phosphorus intake mg. P	Phosphorus output mg. P	Phosphorus balance mg. P
			Oil			
			Female 3, Litter 8			
Preliminary, 7 days	1248	1090	+158	743	529	+214
Experimental, 28 days	4689	4546	+143	2790	2251	+539
			Female 6, Litter 10			
Preliminary, 7 days	1430	1476	−46	852	686	+166
Experimental, 27 days	5330	4798	+532	3173	2504	+669
			Testosterone Propionate			
			Female 17, Litter 8			
Preliminary, 7 days	1165	1125	+40	694	544	+150
Experimental, 28 days	5095	5057	+38	3035	2405	+630
			Female 21, Litter 10			
Preliminary, 7 days	1462	1467	−5	870	691	+179
Experimental, 28 days	5480	4858	+622	3262	2491	+771
			Oil			
			Male 8, Litter 8			
Preliminary, 7 days	1430	1347	+83	852	658	+194
Experimental, 28 days	5330	5542	−212	3173	2825	+348
			Male 11, Litter 10			
Preliminary, 7 days	1398	1443	−45	833	695	+138
Experimental, 27 days	4998	4626	+372	2974	2485	+489
			Diethylstilbestrol			
			Male 23, Litter 8			
Preliminary, 7 days	1808	1527	+281	1076	731	+345
Experimental, 28 days	4480	4447	+33	2665	2243	+422
			Male 26, Litter 10			
Preliminary, 6 days	1159	1276	−117	690	587	+103
Experimental, 28 days	3965	3600	+365	2360	1932	+428

present knowledge. Castration did not serve to accent the action of the injections of testosterone propionate into female rats or diethylstilbestrol into male rats and in both intact and castrated male rats the same decrease in serum acid phosphatase activity was observed. By castration of the male animals an approach has been made toward the duplication of the conditions which exist in treating carcinoma of the prostate. It is still desirable to produce a pathological condition in an experimental animal parallel to the condition of the human with carcinoma of the prostate. The prostate is under the influence not only of the testicular hormones but also the gonadotropic hor-

mone of the pituitary. Therefore, removal of the pituitary or both hypophysectomy and castration might possibly be of greater influence than castration alone. Experiments are already well under way to determine the effect of injecting sex hormones into hypophysectomized rats.

SUMMARY

Daily subcutaneous injections of 0.1 mg. of diethylstilbestrol and 0.2 mg. of testosterone propionate were given castrated male and female rats, respectively, for 28-day periods. There was no change in the serum calcium or phosphorus nor the output of calcium and phosphorus in the feces. As in the intact male rats, diethylstilbestrol produced a decrease in the acid phosphatase activity of the blood serum. Castration did not magnify the effects of the hormone injections. The level of the acid phosphatase activity of the blood serum was raised by castration.

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OPTIMAL MATING TIME FOR PREGNANCY IN THE MONKEY

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THE RHESUS MONKEY (Macaca mulatta) has become the laboratory animal of choice for study in the physiology of reproduction because of the twenty-eight day menstrual cycle, an extended pregnancy (circa 168 days), and the fact that the genital tract, anatomically, is so like a miniature replica of the human. The rhesus baby, too, is born a helpless suckling.

It is the purpose of this paper first to present in summary the results of five years of monkey breeding during which period there was in use a rigorously standardized plan for mating at a particular time in the menstrual cycle and for a limited time only. Second, there will be contrasted with these results the outcome of the following year of breeding in which one change was made in the routine; viz., the time of mating was displaced six days later in the menstrual cycle. The contrast in the results of these two sets of data was reaffirmed by allowing the animals to return to the original routine for a single mating period. Similar human records would be improbable of achievement and these records, therefore, may have a reference value.

The early evidence that monkeys ovulated during midmenstrual cycle from the ninth through the eighteenth days was based on the finding of tubal eggs (Corner, 1923; Allen, 1926) and the occurrence of pregnancy after a single coitus (Hartman, 1932). Hartman's data showed peak days for ovulation to be eleven, twelve, thirteen and fourteen. All timing is, of course, in relation to the first day of preceding menses.

In 1935 systematized breeding was begun in our Obstetric Monkey Colony in order to have a self-reproducing group and to avoid the introduction of tuberculous animals. The colony numbered about sixty animals at that time, more than half of which were young or adolescents. Now, almost all the breeding monkeys, as well as the younger animals, have been born in the colony and there are four generations represented. During the years from 1935 to 1937 the monkeys were mated for the greater part of, or the entire span of,

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ovulation days from the 9th to 18th days in midcycle. From 1937 the duration of mating was narrowed gradually until 1939, when a 48-hour mating period was established as a routine.

The term *mating* when used here simply means that the female has been removed from the living quarters which she shared with a second female and placed in the cage of an adult male. Adult males do not often live amicably together and, therefore, are caged separately. The 48-hour mating, used routinely since 1939, began most often on the eleventh day of the menstrual cycle. Successful impregnation, however, followed matings on days nine, ten, eleven, and twelve, six pregnancies being recorded for day nine and three for day twelve.

Analysis of all the 48-hour matings which resulted in pregnancy disclosed the fact that the 24-hour period common to the greatest number of matings extended from noon of the eleventh day to that of the twelfth (van Wagenen, 1944). Here it should be explained that, for reasons of accuracy in records and timing, all changes of animals

TABLE 1. SUMMARY OF 11TH-DAY MATINGS NECESSARY FOR PREGNANCY IN PRECEDING FIVE YEAR PERIOD

	Single mating	Two matings	Three or more matings	Total number of pregnancies for year
1943	6	4	12	22
1942	6	8	4	18
1941	7	6	8	21
1940	10	6	14	30
1939	8	4	12	24
totals	37 (32%)			115 (100%)

took place at noon. In the case of an eleventh-day mating, the female was placed in the cage of a male at noon on the eleventh day of her menstrual cycle and removed on the second day thereafter at noon.

Although theoretically a monkey can have three babies in two years, it has been customary in our colony to expect only one full-term (six months) pregnancy a year. The routine has been to mate the monkeys in the fall months, beginning September 1st, allowing the preceding summer to be a rest period with no nursing young. The newborn monkey was generally removed at once from the mother, but there were a few instances of nursing for one or two months. Protracted nursing was never allowed, so that breeding monkeys were living apart from their young and with animals of their own sex during the summer months preceding the arbitrarily imposed season of mating.

Table 1 reviews one phase of five years of monkey breeding. During this time there was a total of one hundred and fifteen pregnancies and they have been examined in an effort to evaluate the success of the 11th-day mating. That is, the question was asked—how many times was mating necessary before conception took place? The results

were similar for each year and it was found that roughly a third of the pregnancies for a year followed a single mating. Examining the totals, 32% became pregnant after one mating and 24% at the next mating, so it can be said that more than half the animals (56%) became pregnant at a first or second mating. In the case of multiparous animals all matings since the birth of the last baby were counted; and, in the young nulliparous animals the matings were reckoned from the third year of age. As would be expected, multiparous animals required fewer matings and the greatest parities usually were found in the single mating group.

Monkeys of low fertility, or possibly sterile, have been encountered, one or two a year, but, since fertility rather than sterility was the subject of interest, these animals were assigned to other experiments if, after frequent rectal palpation during the fall months, it was found that the ovaries and uterus remained relatively small and unchanging. Also, uncooperative animals were apprehended and ultimately discarded.

To circumvent factors limiting successful impregnation, multiparous animals were placed with the fathers of their last babies and nulliparas with a preferred male or one not obviously antagonistic. As suggested earlier, unfavorable times for conception were for the most part avoided. Young animals were not mated during puberty; multiparous animals were not mated within three months after a full-term pregnancy, even if menstrual cycles had resumed; and, too, mating rarely was planned for the summer months, when anovulatory cycles are characteristic of this animal. So, in this study, selected animals were mated at favorable times in relation to age, preceding pregnancy and lactation. They were mated on the 11th day of the menstrual cycle and the results for each of five years were consistently similar (Table 1).

Since the success of the 11th-day mating had been evaluated by the finding that *one third of the pregnancies followed a single mating*, the next step seemed to be the selection of a different mating time in the menstrual cycle for comparison of results. In 1944 the initial day of mating was changed. Twenty-five adult female monkeys (22 multiparas and 3 nulliparas) were available for breeding and beginning September 1st all matings were planned for the 17th day of the cycle. The females remained in the mating cage the usual 48-hour period. Table 2 indicates the number and relative time arrangement of the 17th-day matings during the four months—September to January first. In some cases postgestational amenorrheas and long summer cycles extended into the early fall months and account for the scattered matings on the left of the table.

In the preceding years, with the 11th-day mating, all but the exceptional animal or two would have become pregnant by the end of the year and the first babies were expected to appear early in

March. But, with the 17th-day mating, only one animal, Monkey 00, was pregnant in December. The menstrual history of this animal will be examined later. During the four months, September to December, the twenty-five animals were mated 80 times. Monkeys 22, 27, and 51 were mated twice each, Monkeys 41 and 97 five times, and the other twenty-one animals either three or four times. Here, the result of one pregnancy in twenty-five animals after eighty matings was in great

TABLE 2

1944 Animal	17th-day matings Sept.	Oct.	Nov.	Dec.		11th-day matings Jan.	
72		m	m	m		m	
92		m	m	m		m	
17	m		m	m		m	
96	m	m	m	m		m	
04		m	m	m		m	preg
16	m	m	m	m		m	preg
22			m	m		m	
41	m	m	m	m		m	
42		m	m	m		m	preg
47			m	m		m	
48	m		m	m		m	
51			m	m		m	preg
70		m	m	m		m	
73		m	m	m		m	
74		m	m	m		m	
82		m	m	m		m	
85	m		m	m		m	
[1] 00	m		m	m	pregnant		
[1] 01			m	m		m	preg
[1] 07			m	m		m	preg
[1] 18		m	m	m		m	
20	m			m		m	
27			m	m		m	
63			m	m		m	
97	m	m	m	m		m	preg
Total number of matings and pregnancies				80	1	24	7

m—mating
[1] See Fig. 1 for analysis of menstrual cycles and mating of these animals

contrast to the reproductive performance of the same animals in the preceding years.

On January first the 11th-day mating was resumed. Of the twenty-four monkeys, seven were pregnant following a single mating. This approached the record of the preceding years, confirming the ratio of mating to pregnancy when mating takes place on the eleventh day. The January pregnancies, of course, carry on into June and, since it was desirable to have the monkeys free from pregnancy in the summer months in order that they might be available for reproductive studies in September, repeated breeding after January was undesirable.

Since Monkey 00 became pregnant under conditions that were not favorable for pregnancy in her twenty-four associates, her menstrual record was examined. Figure 1 pictures this record together with the menstrual records of the three animals following her in sequence;

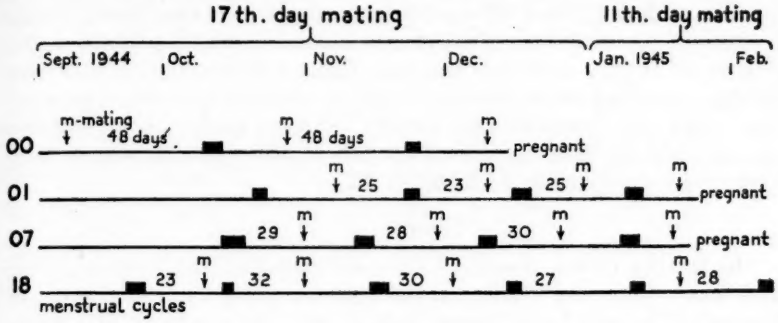

FIG. 1

Monkeys 01, 07, 18. The different character of cycle length in Monkey 00 is at once apparent. The two 48-day cycles, followed by a 37-day cycle (Table 3), indicate a departure from the usual deviation in cycle

TABLE 3

1944 Animal	Sept.		17th-day mating Oct.	Nov.	Dec.
72		41		29	27
92			29	29	28
17	59			28	28
96	36	51		27	27
04			29	27	29
16	64		29	26	27
22				30	28
41	33	28	24	24	26
42			29	27	33
47				29	57
48	64			29	29
51				28	22
70			26	26	29
73		28	28	28	27
74			27	27	29
82		24	27	25	23
85	46			27	26
00	48		48		37
01			28	28	25
17			29	28	30
18		23	32	30	
20	87			28	25
27			30	29	28
63			28	24	26
97	31	26	26	26	26

Note: To be compared with Table 2.

length of the rhesus monkey. To the 48-day cycles of Monkey 00 one can compare the eleven cycles of the other three monkeys in Figure 1, which have an average length of 27 days, the longest cycle being 32 days. The 17th-day mating appears to have coincided sufficiently well with ovulation in the longer cycle, bringing about the pregnancy in Monkey 00.

The cycle lengths of all the other monkeys were examined to learn if similar long cycles occurred in any animal in which pregnancy did not result from a 17th-day mating. Table 3 shows that cycles over 40 days occurred seven times in as many animals but, when present, they were not repeated and usually followed gestation or summer amenorrhea, so that there was no other monkey with consistently long cycles comparable to Monkey 00.

SUMMARY

In mating rhesus monkeys (Macaca mulatta) the routine procedure was to place the female, on the *11th day* of her menstrual cycle, in the cage of a male for a period of 48 hours. The results of five years, 1939 through 1943, of this program were reviewed and it was shown that in each year roughly one third of the pregnancies followed a single mating.

In 1944 the initial mating day was delayed to the *17th day* of the menstrual cycle, while all other procedures were unchanged. Twenty-five animals were mated more than an average of three times each, eighty times in all, but at the end of four months only one pregnancy had been effected. In contrast, when the remaining 24 monkeys then were mated on the *11th day* of their next cycle, seven monkeys became pregnant.

It was concluded that *mating opportunity on the 17th day of the menstrual cycle is too late in relation to the time of ovulation and viability of the ovum in this monkey.* The eleventh to thirteenth days of the menstrual cycle remain the preferred days for mating.

ACKNOWLEDGEMENT

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GLUCOSE TOLERANCE IN RATS FOLLOWING REPEATED SMALL DOSES OF ALLOXAN

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IT HAS BEEN SHOWN conclusively that a single injection of a large dose of alloxan (200 to 400 mg./kg.) administered subcutaneously produced severe and permanent diabetes in rats by causing necrosis of beta cells in the pancreatic islets (Dunn, Sheehan and McLetchie, 1943; Goldner and Gomori, 1943). Single intravenous injections of 60 to 75 mg./kg. produced equally severe diabetes in rats (Shipley and Meyer, unpublished data).

The effect of small repeated doses of alloxan on the functional capacity of the islet tissue is not so certain. Thorogood (1944) found that four out of six rats developed glycosuria within four weeks when daily subcutaneous injections of alloxan (50 mg./kg.) were administered. This same dose intravenously administered daily to rabbits by Kennedy and Lukens (1944) produced diabetes, but 25 mg./kg. doses repeated daily did not. Thus it would appear that in sufficiently large doses alloxan has a cumulative diabetogenic effect. On the other hand Hughes and Hughes (1944) concluded from their studies that prolonged repeated administration of small doses of alloxan led to the destruction of only the older beta cells and accelerated maturation of the younger cells of the pancreatic islets, although they reported extensive damage from 125 mg./kg. daily doses over a two months period.

The purpose of this investigation was to determine whether the functional response of the pancreatic islets to the administration of glucose was altered by the repeated administration of small doses of alloxan.

MATERIALS AND METHODS

Group 1. A 2.5% alloxan solution was used in all cases. The rats of this group were female albinos. They were 35 days of age at the beginning of the experiment, and weighed 52 to 73 grams. Intravenous injections of alloxan in 25 mg./kg. equivalents were given to 10 rats every second day for a period of 4 weeks, so that the experimental rats received a total of 14 doses. Controls were 7 rats of the same age and weight, but were uninjected. Blood glucose determinations were made on the normal controls and the experimental

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animals before the injections were begun and just before each alloxan injection was administered. Glucose tolerance tests were made on these animals at varying periods after the series of injections was stopped.

Group 2. Both male and female rats were included in this group, but the animals were divided so that equal numbers of each sex and of each litter were included in the experimental and the control group. The experimental animals of the group were given 25 mg./kg. doses of 2.5% alloxan at intervals of 1 to 3½ weeks and they were given a glucose tolerance test after each injection, until a total of six sub-diabetogenic doses of alloxan had been administered.

Glucose tolerance test: For these tests the technique of Cole and Harned (1938) was employed. The glucose was administered by intraperitoneal injection, after the animals had been fasted overnight (16 to 18 hrs.). The dose of glucose was 3.5 gm./kg. of body weight, or 3.5 cc. of 10% glucose per 100 gm. of body weight. Blood glucose determinations were made in the fasting animals and at ½, 1, 2, 3 and 5 hours after glucose administration. Blood samples were obtained from the tip of the tail. Blood glucose determinations were made colorimetrically according to the method of Reinecke (8).

RESULTS AND DISCUSSION

The blood glucose levels in the rats of group 1, given 14 successive small does of alloxan, are summarized in Table 1. Ten experimental

TABLE 1. NON-FASTING BLOOD GLUCOSE IN RATS OF GROUP 1 DURING THE PERIOD OF TREATMENT WITH SUBMINIMAL DOSES OF ALLOXAN

Rats	Blood glucose in mg./100 cc.														
	Number of alloxan injections														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	144	139	153	170	139	140	159	160	142	141	144	135	140	137	109[1]
Experimental	154	149	145	172	159	138	158	153	149	154	149	146	151	139	118[1]

[1] Fasting level.

animals and 7 controls were used in this experiment; however some of the rats were killed accidentally and the later determinations were made on fewer rats. The blood glucose levels in Table 1 were determined on animals which had not been fasted except for the last determination. It can be seen that, although there were fluctuations in values from day to day, there were no significant differences in the blood glucose levels found in the two divisions, under the normal conditions of feeding.

Figure 1 represents the average growth of the injected and uninjected rats during the period of injections. There was no essential difference in their weights at any time during the period of injections, so that it can be assumed the alloxan treatment did not interfere with the rate of growth or the general welfare of the rats.

Two days after the last injection of alloxan the rats were fasted overnight and given a glucose tolerance test the following day. Table 2 gives the average fasting blood glucose level and the average blood glucose level in both control and experimental animals for each of the periods tested. The highest levels were one-half hour after the glucose administration and at that period there was approximately 100 mg./100 cc. difference between the blood sugar levels in the experimental and control animals. The treated rats also exhibited the slower return to normal that was seen in partially diabetic rats (Cole and Harned, 1938). Figure 2 shows the difference in the glucose tolerance of the two groups immediately after the treatment was stopped.

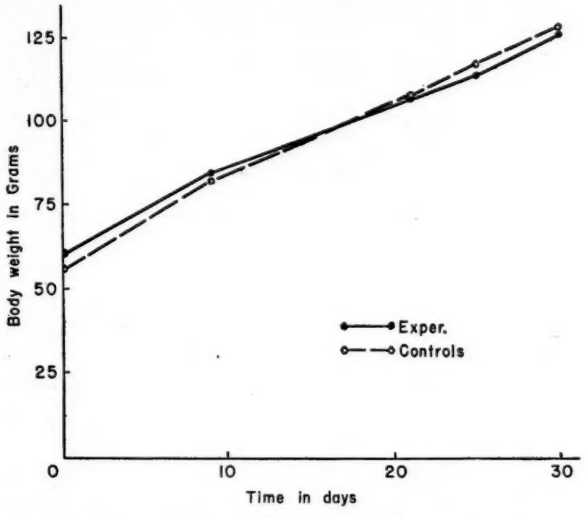

FIG. 1. Growth of rats in group 1 while receiving 25 mg. of alloxan per kg. of body weight, every second day.

To eliminate any differences in glucose tolerance which might be only transitory, the rats were tested again for impairment of glucose tolerance at periods of 2, 4, 6, and 8 weeks after cessation of treatment with alloxan. The blood glucose levels for each of these periods can be seen in Table 2. The data in Table 2 clearly indicate that impairment of glucose tolerance was not transitory. It would suggest that any maturation of beta cells after necrosis of older ones, as Hughes and Hughes (1944) found, was not adequate to restore pancreatic function to normal.

It is conceivable that repeated glucose tolerance tests might overtax an already damaged pancreas, so that further damage might ensue as a direct result of the glucose treatment. It was decided that

TABLE 2. PERSISTENCE OF IMPAIRMENT OF GLUCOSE TOLERANCE AFTER ALLOXAN

Time interval after alloxan	Division	Hours after glucose administration					
		0	½	1	2	3	5
		Glucose in mg./100 cc. of blood					
2 das.	C	108.9	274.8	180.5	154.9	161.1	127.1
	E	117.7	374.9	364.8	250.3	186.1	138.4
2 wks.	C	108.5	222.5	186.5	164.1	144.5	127.4
	E	115.8	333.4	288.4	188.8	149.5	119.6
4 wks.	C	103.0	250.7	167.0	147.9	140.2	130.5
	E	106.7	321.4	262.4	199.6	174.0	142.4
6 wks.	C	107.6	218.4	172.8	146.2	137.6	149.0
	E	101.7	355.1	331.2	208.7	201.0	201.8
8 wks.	C	94.9	246.2	156.2	156.2	134.9	128.1
	E	96.0	352.9	325.8	197.9	147.3	117.0
15 wks.	C	110.2	243.4	168.8	153.2	148.5	143.7
	E	129.9	316.2	265.5	205.4	219.3	174.8
23½ wks.	C	106.1	281.6	187.4	162.3	148.5	117.5
	E	101.4	375.4	336.3	277.8	240.5	166.3

C = Control.
E = Experimental.

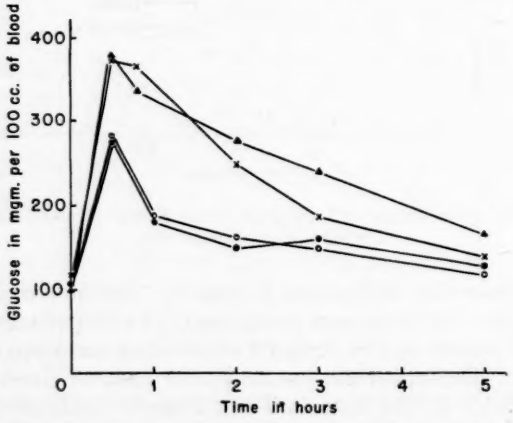

FIG. 2. Glucose tolerance in rats given fourteen successive small doses of alloxan.
●——● average blood glucose in control rats 2 days after the last alloxan treatment.
x——x average blood glucose in experimental rats 2 days after the last alloxan treatment.
○——○ average blood glucose in control rats 23½ weeks after the last alloxan treatment.
▲——▲ average blood glucose in experimental rats 23½ weeks after the last alloxan treatment.

a longer interval should be allowed before another tolerance test was made so that any repair of islets would be unimpeded. Accordingly, a period of seven weeks, was allowed to elapse, and at 15 weeks after the last alloxan treatment the rats were given another glucose tolerance test. Table 2 shows that the impairment to glucose tolerance in these rats was still of the same order of magnitude as was seen in earlier tests. One other glucose tolerance test was given at $23\frac{1}{2}$ weeks after the last alloxan administration and Table 2 includes the blood glucose values found in that test. Figure 2 also shows the curves for the control and experimental animals for the last test. It will be seen that both control and experimental blood sugar values are approximately the same as those obtained immediately after the last alloxan injection, and it may be assumed that adequate repair or multiplication of beta-cells did not follow treatment with small doses of alloxan.

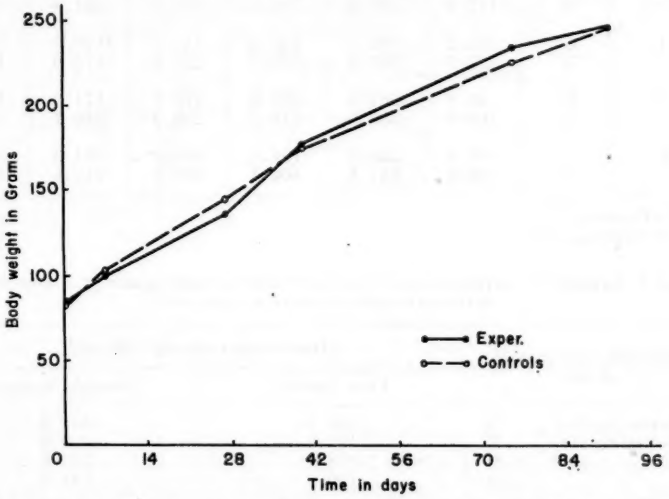

FIG. 3. Growth of rats of group 2 given six injections of alloxan in 25 mg./kg. dose equivalents during a period of 91 days.

It seemed pertinent to the question of the extent of damage from single sub-diabetogenic doses of alloxan to determine the response of rats to glucose administration after each small dose of alloxan, in order to see if progressive impairment could be demonstrated. For this experiment the rats of group 2 were used. The group was composed of male and female rats, but they were equally divided as to litter and sexes between the control and experimental divisions, so that the composition of the two divisions were comparable. The rats were immature and ranged from 65 to 104 grams in weight at the beginning of the experiment. Figure 3 shows the growth in the control and experimental divisions and it can be seen the alloxan treatment did not

inhibit the growth of the rats. The alloxan injections were made at widely spaced intervals of 1 to 3½ weeks, and the glucose tolerance tests were made two days after the corresponding alloxan injection,

TABLE 3. IMPAIRMENT OF GLUCOSE TOLERANCE AFTER SUCCESSIVE DOSES OF ALLOXAN

No. of doses of alloxan	Division	Hours after glucose administration					
		0	½	1	2	3	5
		Glucose in mg./100 cc. of blood					
1	C	95.4	218.9	158.0	130.2	104.8	109.8
	E	101.0	194.1	181.0	138.8	112.8	106.5
2	C	119.5	187.0	137.4	125.9	120.3	106.7
	E	119.3	215.6	182.9	166.3	142.9	107.8
3	C	105.4	200.5	142.3	126.7	120.2	122.3
	E	117.2	268.3	215.3	182.7	131.8	114.8
4	C	104.2	182.5	153.4	131.3	128.0	126.1
	E	117.2	303.0	268.1	211.2	172.0	135.6
5	C	98.2	282.9	220.6	170.7	171.6	150.7
	E	105.9	383.5	379.3	296.9	256.1	220.5
6	C	93.8	266.5	191.5	158.1	154.3	127.8
	E	88.8	354.8	404.4	299.1	245.4	194.6

C = Control.
E = Experimental.

TABLE 4. EFFECT OF ANTERIOR PITUITARY EXTRACT ON THE BLOOD GLUCOSE OF RATS WITH IMPAIRED GLUCOSE TOLERANCE

Treatment and Number of rat		Blood sugar in mg./100 cc.[1]	
		First Sample	Second Sample
Alloxan treated followed by APE	3	118.5	332.5
	7	112.5	430.5
	9	118.0	206.0
	10	105.5	158.5
Average		113.6	281.8
Control untreated	2	96.5	144.5
	3	132.5	131.0
	4	130.5	130.0
	5	96.5	131.0
	6	105.5	138.0
Average		112.3	112.4

[1] Not fasting.

except in one instance (after the 5th dose) when the tolerance test was postponed until 14 days after the injection of alloxan.

Table 3 is a summary of the blood glucose levels found for the control and for the experimental rats in the glucose tolerance tests given after each alloxan treatment. Figure 4 shows clearly the prog-

ress of the damage to the pancreatic islets after the administration of successive small doses of alloxan. The lines of the graph represent the differences in blood sugar levels in the experimental group and in the control blood glucose levels for any given test. It was believed that this representation was more accurate than if the control values were averaged, since it allowed for differences in physiological factors such as temperature, ageing, and other variables. The glucose tolerance curves after the first subdiabetogenic dose of alloxan suggest damage

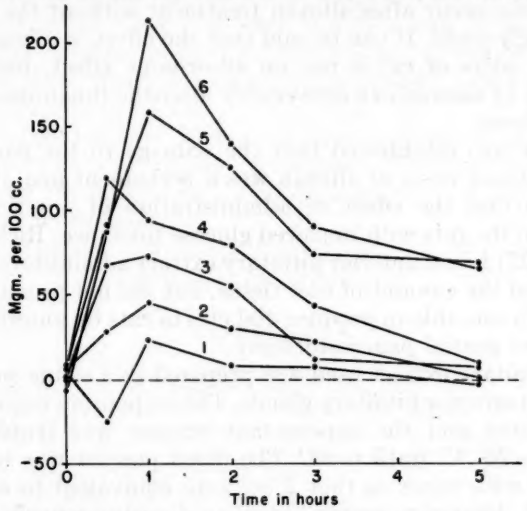

FIG. 4. Glucose tolerance in rats of group 2. Lines 1 to 6 represent the difference in blood glucose values of the alloxan treated rats and their controls after each small dose of alloxan.

since the injected group was a little slower in returning to normal levels, but such a slight difference in response was equivocal. Following the second dose of alloxan, which was given one week after the first dose, there was no doubt but that damage had been done to the insulin-producing cells since the blood glucose of the injected group rose higher and executed a slower return to normal levels than in control rats. The curves seen in Figure 4 demonstrate a definite increase in pancreatic damage following each dose of alloxan. The increases in levels of blood glucose are apparent, and the slower return of blood sugar to normal levels in the treated rats are equally striking in the degree of change which followed the injections.

From the experiments with the two groups of rats we may conclude that even sub-diabetogenic doses of alloxan exert a damaging effect upon the insulin-producing cells in rats, and that the damage is cumulative in nature, as was found by Kennedy and Lukens (1944) for rabbits. If a maturation of beta-cells occurred after repeated injections of alloxan, as was proposed by Hughes and Hughes (1944),

the cells were not physiologically equivalent to those which composed the islets before alloxan treatment. Since there was no return to normal functioning for 23½ weeks after the last injection of alloxan, the indications are that such treatment led to progressive and accumulative damage to islet tissue. None of the rats used in these experiments became diabetic to the extent of showing a hyperglycemia under normal conditions of feeding. Our results seem to be of value because of the demonstration that functional impairment of glucose tolerance can occur after alloxan treatment without the appearance of a hyperglycemia. It can be said that the effect of alloxan upon the pancreatic islets of rat is not an all-or-none effect, but that even small doses of alloxan can irreversibly alter the functional reserves of the islet tissue.

After it was established that the damage to the pancreas from successive small doses of alloxan was a permanent one, it became of interest to find the effect of administration of anterior pituitary extract into the rats with impaired glucose tolerance. Richardson and Young (1937) found anterior pituitary extract administered to normal rats doubled the amount of islet tissue, but did not produce diabetes. Long (1937) was able to produce diabetes in rats by anterior pituitary extract after partial pancreatectomy.

The pituitary extract used was prepared as a saline suspension of ground beef anterior pituitary glands. The suspension was centrifuged, then decanted and the supernatant extract was lyophilized and stored at −20° C. until used.[1] The dried powder was made into a suspension with water so that 2 cc. were equivalent to one gram of fresh gland. Four rats previously treated with fourteen small doses of alloxan were given 1 gm. equivalents per day of the extract by subcutaneous injection, and the blood glucose values of the injected rats and of control rats were determined after the eleventh day of administration. Table 4 gives the individual and the average blood glucose values in non-fasted rats before any anterior pituitary extract (APE) was given, and on the twelfth day, after eleven doses of anterior pituitary extract. It can be seen that the blood glucose values in the rats with impaired glucose tolerance were essentially the same before APE administration as the values in the control rats, but after APE was given the blood sugar values were increased in all the injected rats and markedly increased in all except rat #10.

The data seem to warrant the conclusion that the series of small doses of alloxan had produced a condition equivalent to partial pancreatectomy and that the remnant of islet tissue was unable to respond to the increased demands upon it when APE was given. The study with impaired glucose tolerance and APE is being extended and will be published in more detail at a later time.

[1] We are indebted to Dr. Leo Ciereszko of the Biochemistry Department, Medical-Research Division, Sharp and Dohme, for the preparation of the lyophilized anterior pituitary extract.

SUMMARY

Rats given 14 sub-diabetogenic doses of alloxan over a period of four weeks did not become hyperglycemic on the normal diet, but showed impaired tolerance to administered glucose.

The decrease in tolerance to glucose appeared to be permanent since it remained for 23½ weeks after cessation of alloxan treatment.

In a second group of rats there was a marked and progressive decrease in glucose tolerance after each injection of a 25 mg./kg. dose of alloxan. The decrease in glucose tolerance was shown by the higher blood glucose levels reached early in the tests, and in the slower return of the glucose levels to normal.

It was concluded that 25 mg./kg. doses of alloxan given intravenously produced a progressive damage to pancreatic islets in rats, and that even the small amount of damage was not followed by a compensatory mechanism.

Anterior pituitary extract administered to four rats with impaired glucose tolerance produced hyperglycemia after 11 days, in 3 of the 4 animals.

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METABOLISM OF THE STEROID HORMONES: THE ISOLATION OF Δ^5-ANDROSTENEDIOL-3(β),17(α) AND Δ^5-PREGNENEDIOL-3(β),20(α)

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MARKER AND ROHRMAN (1938) isolated Δ^5-pregnenediol-3(β),20(α) from the urine of pregnant mares. This steroid and Δ^5-androstendiol-3(β),17(α) have been isolated recently from the urine of a boy suffering from an adrenocortical carcinoma by Hirschmann and Hirschmann (1945). The isolation is reported here of Δ^5-pregnenediol-3(β),20(α) and Δ^5-androstenediol-3(β),17(α) from the urine of a woman suffering from an adrenocortical carcinoma, and of Δ^5-pregnenediol-3(β),20(α) from the urine of a girl suffering from virilism.

PATIENT A

The patient with an adrenal cortical cancer was thirty years old. When first seen at the clinic her chief complaints were amenorrhea of seven months' duration, increased growth of body hair and frequent headaches for five months. Menarche occurred at the age of thirteen; menstrual periods being regular and normal until fifteen months prior to our examination. Since that time her menstrual periods were irregular and frequent. Hypertrichosis involved the forearms, adbomen, thighs, surface of the breasts and recently there had appeared a noticeable mustache but no hypertrichosis of consequence on the face. The skin was oily. The headaches were bilateral and temporal and could be relieved readily with aspirin. During the past six or seven months, there had been some atrophy of the breasts and the voice

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had become distinctly low and coarse. Examination showed features suggestive of masculinity. Musculature of the arms and shoulder girdles were also prominent. There was some recession of the hairline in the temporal area. Her demeanor, however, was feminine. Pelvic examination revealed some enlargement of one ovary, the other having been removed some years ago.

A vaginal smear showed chiefly leucocytes and deep cells. The clitoris was markedly hypertrophied. The uterus was thought to be normal in size. The sella turcica was normal to x-ray.

For several months following the removal of an adrenal cortical tumor, the patient felt relatively well. Her weight had never varied but a few pounds from the time of the first examination. Three months postoperatively, there was evidence of local recurrence of the tumor. From that time on she went steadily downhill. Liver enlargement was observed at this time, and she became rapidly weaker and died six months after the operation. Autopsy showed a cortical carcinoma of the adrenal, recurrent at the site of excision of the original tumor, metastasis to the right kidney which was enlarged to about four times its normal size, and multiple metastases to the liver. The largest metastasis measured 10 cm. in diameter and was located in the right lobe of the liver. It had a homogenous appearance and was approximately the color of the yolk of a hard boiled egg. The left adrenal gland weighed 9 grams and was normal to inspection externally and on cut section.

PATIENT B

The girl suffering from virilism (Wisconsin General Hospital No. 127483) has been described in a previous publication (Dorfman, Schiller, and Sevringhaus, 1945). She showed slight hirsutism on the upper lip and chin, lack of mammary development, masculine hair pattern on the lower abdomen and about the pubis, and a marked enlargement of the clitoris. At laparotomy the adrenals seemed normal to palpation, but the ovaries, although of normal size, were polycystic in type. Biopsies of the ovaries showed dense stroma and a number of small follicles.

EXPERIMENTAL

Patient A, Adrenal Cortical Cancer

Two samples of urine were collected from Patient A and the neutral non-ketonic fractions studied. The first sample of 12 liters was collected about twenty weeks before death. The urine was acidified with 15%, by volume, of concentrated hydrochloric acid and refluxed for 6 hours with $\frac{1}{4}$ volume of carbon tetrachloride. The carbon tetrachloride layer was separated and the acidified urine reflexed for an additional 6 hours with $\frac{1}{4}$ volume of fresh carbon tetrachloride. The combined carbon tetrachloride layers were concentrated to dryness in vacuo, taken up in ether, and the acidic and phenolic compounds removed with 10% sodium hydroxide.

The neutral fraction remaining in the ether layer was separated with the Girard-Sandulesco reagent T (Dorfman, Cook, and Hamilton, 1939). The ketonic neutral fraction assayed 114 I.U. per liter by the chick comb method for androgens (Frank et al., 1943) and 160 mg. of 17-ketosteroids by the Holtorff and Koch (1941) method.

The neutral non-ketonic fraction was treated with digitonin in 90% ethanol, so that the final digitonin concentration was 1%. Attempts to isolate pure substances from the β-hydroxy non-ketonic neutral fraction by chromatographic analysis of the free compounds using aluminum oxide failed. The various eluates were recombined, evaporated to dryness and the residue acetylated with acetic anhydride and pyridine. The acetates, weighing 168 mg., were dissolved in 15 cc. of a mixture of petroleum ether (4 parts) and benzene (1 part) and adsorbed on 10 gm. of aluminum oxide. The first two elutions with 50 cc. of a mixture of petroleum ether (3 parts) and benzene (1 part) yielded crystalline material which melted at 137–140° C.[1] Treatment of this compound with alcoholic sodium hydroxide yielded 5 mg. of a compound melting at 175–178° C. When this compound was mixed with an authentic sample of Δ^5-pregnenediol-3(β),20(α) (M.P. 180–183° C.)[2] the melting point was 175–179° C.

The second sample of urine (3.2 liters) was collected from the same patient about two weeks before death. The sample was acidified with 10% hydrochloric acid by volume and extracted three times with ¼ volumes of n-butanol. The butanol extractions were combined and concentrated to about 1 liter. The butanol solution was permitted to stand at room temperature for about one month. A small sample (about 1/30 of the total) of the butanol solution was taken to dryness and the residue partitioned between 100 cc. of ether and 100 cc. of water. The ether layer was extracted further with two 100-cc. portions of water, three 25-cc. portions of 10% sodium hydroxide, and finally with water. The ether layer contained 225 mg. of 17-ketosteroids. The water layers of the ether-water partitions were combined, acidified with 15% hydrochloric acid by volume and extracted with carbon tetrachloride as described previously. The neutral fraction yielded 4.4 mg. of 17-ketosteroids. Thus 98% of the 17-ketosteroids were present in the free form.

The total butanol extract was evaporated to dryness in vacuo, dissolved in 400 cc. of ether and extracted three times with 100 cc. portions of 10% sodium hydroxide, and finally with water. The ether was evaporated and the residue treated with Girard-Sandulesco reagent T.

One-fourth (equivalent to 0.8 liter of urine) of the non-ketonic neutral fraction (3.1 gm. residue) was separated twice with digitonin in 90% ethanol. The digitonin precipitable fraction yielded 536 mg.

[1] All melting points are uncorrected and were taken with the Fischer-John apparatus.

[2] The sample of Δ^5-pregnenediol-3(β),20(α) was kindly furnished by Dr. H. Hirschmann.

in the first precipitation and an additional 31.7 mg. in the second precipitation, or a total of 567.7 mg. After acetylation with acetic anhydride and pyridine, the residue weighed 602.7 mg. The acetylated product was dissolved in 9 cc. of a mixture of benzene (1 part) and of petroleum ether (2 parts), and adsorbed on 15 gm. of aluminum oxide (Brockmann). The column was developed with four 50 cc. portions of benzene (1 part) and petroleum ether (2 parts). The residue from the first fraction consisted of a heavy oil which crystallized on standing, but which could not be purified further by recrystallization. The material was dissolved in a mixture of benzene (1 part) and petroleum ether (5 parts) and adsorbed on a column of aluminum oxide. Elutions were carried out with successively increasing concentrations of benzene in petroleum ether, benzene, and finally with benzene-ether mixtures. The early benzene eluates yielded a crystalline residue from which, after repeated recrystallization from absolute methanol, 14.8 mg. of a substance with a constant melting point of 158–160° C. was obtained. Later benzene eluates, which were recrystallized from absolute methanol, yielded 3.2 mg. of a compound with a melting point of 161–163° C. A mixture of the two substances melted at 159–162° C.

A sample of the compound melting at 158–160° C. was dried at 100° C. and 1 micron for 1 hour.

Analysis—$C_{23}H_{36}O_3$ (M.W. 360.52)—Calculated C = 76.62 H = 10.07
Found C = 75.76 H = 9.82

After treatment of a sample of the compound which melted at 158–160° C. with sodium hydroxide in an aqueous ethanolic solution, Δ^5-pregnenediol-3(β),20(α), M.P. 177–179° C. was isolated. There was no depression of the melting point when it was mixed with an authentic sample of Δ^5-pregnenediol-3(β),20(α). Therefore the compound, melting point 158–160° C. was Δ^5-pregnenediol-3(β),20(α) monoacetate.

Eluation with a mixture of benzene (4 parts) and ether (1 part) yielded crystalline material. After repeated recrystallization from absolute methanol, 5.0 mg. of a substance melting at 168–170° C. was obtained. A portion of this material was saponified and yielded a compound with a melting point of 175–176° C. When mixed with an authentic sample of Δ^5-pregnenediol-3(β),20(α) the mixture melted at 175–177° C. A mixture of the unsaponified substance with that obtained after saponification melted at 169.5–174° C. Another portion of the material was treated with pyridine and acetic anhydride. The diacetate of Δ^5-pregnenediol-3(β),20(α) was obtained. It seems likely that the substance obtained from the benzene-ether eluates was the free pregnenediol probably contaminated with a small amount of the monoacetate.

A sample of Δ^5-pregnenediol-3(β),20(α), which was recovered after saponification of the monoacetate, was recrystallized from absolute

methanol (M.P. 177–178° C.) and was dried at 100° C. and 1 micron for 1 hour.

Analysis—$C_{21}H_{34}O_2 \cdot 1/2CH_3OH$ (M.W.—334.50)
Calculated C = 77.19 H = 10.85
Found C = 77.06 H = 10.77

Fractions 2, 3, and 4 were combined and purified by recrystallizing from absolute methanol and 95% ethanol. Three crops of Δ^5-androstenediol-3(β),17(α) diacetate were obtained. The first crop weighed 14.2 mg. and had a melting point of 154–156° C. No depression in melting point was observed when mixed with an authentic sample of Δ^5-androstenediol-3(β),17(α) diacetate (M.P. 158–158.5° C.).[3]

A sample was dried at 100° C. and 1 micron pressure for 1 hour.
Analysis—$C_{23}H_{34}O_4$ (M.W. 374.50)—Calculated C = 73.76 H = 9.15
Found C = 74.00 H = 9.30
M.W. = 378, 382

The second crop of Δ^5-androstenediol-3(β),17(α) diacetate consisted of 14.4 mg. melting at 152–154° C. No depression was noted when mixed with an authentic sample of Δ^5-androstenediol-3(β),17(α) diacetate. After saponification the free compound (M.P. 176–178° C.) was obtained. It did not depress the melting point of an authentic sample of Δ^5-androstenediol-3(β),17(α). A third crop of 12.8 mg. of the diacetate of Δ^5-androstenediol-3(β),17(α) (M.P. 151–154° C.) was isolated and identified. Thus the total yield of Δ^5-androstenediol-3(β),17(α) diacetate amounted to 41.4 mg.

Patient B, Virilism

A total of 25.4 liters of urine was collected. The urine was acidified with 10% concentrated hydrochloric acid heated under the reflex for 15 minutes and immediately cooled under the tap. The hydrolyzed urine was extracted three times with ¼ volume of benzene. The combined benzene extracts were evaporated to dryness. The residue was dissolved in ether and extracted with 10% sodium hydroxide and finally washed with water. The total neutral fraction was separated with the Girard-Sandulesco reagent T and the neutral non-ketonic fraction separated with digitonin. The β-hydroxy non-ketonic neutral fraction (120 mg.) was acetylated with acetic anhydride and pyridine and the acetates adsorbed on 4 grams of aluminum oxide from 50 cc. of petroleum ether. The column was eluted with 50 cc. portions of mixtures of benzene and petroleum ether, pure benzene and a mixture of benzene and ethyl ether. Fractions 9 through 15, all 50 cc. portions of petroleum ether (9 parts) and benzene (1 part), yielded crystalline material. Fraction 12 yielded 3 mg. of a substance, M.P. 142–145° C., which when mixed with an authentic sample of Δ^5-pregnenediol-3(β),20(α) diacetate (M.P. 144° C.) melted at 141.5–144° C.

[3] The sample of Δ^5-androstenediol-3(β),17(α) was kindly furnished by Ciba Pharmaceutical Products, Inc., Summit, N. J.

Fraction 34, which was eluted with 50 cc. of a mixture of benzene (9 parts) and ethyl ether (1 part) after recrystallizing from methanol and ethanol, yielded 2.3 mg. of a substance, M.P. 157.5–161° C. When mixed with an authentic sample of Δ^5-pregnenediol-3(β),20(α) monoacetate (M.P. 158–160° C.) the melting point was 159–160° C. The monoacetate was further acetylated with acetic anhydride and pyridine and yielded the diacetate, M.P. 143.5–144° C., which did not depress the melting point of an authentic sample.

DISCUSSION

The presence of a high concentration of Δ^5-androstenediol-3(β),17(α) in the urine of the patient suffering from an adrenal cortical cancer is of interest because of its close relationship to dehydroisoandrosterone. This particular patient also excreted what is perhaps

TABLE 1. EXCRETION OF Δ^5-PREGNENEDIOL-3(β),20(α) AND Δ^5-ANDROSTENEDIOL-3(β),17(α)

Patient		Δ^5-Pregnenediol-3(β),20(α) Mg./Liter	Δ^5-Androstenediol-3(β),17(α) Mg./Liter
A. Adrenal Cortical Cancer	20 weeks before death	0.33	—
	2 weeks before death	24.5	48.0
B. Virilism		0.18	—

the highest recorded amount of dehydroisoandrosterone. It was possible to isolate 702 mg. of this androgen per liter (Dorfman and Horwitt, 1945). A further direct relationship between Δ^5-androstenediol-3(β),17(α) and dehydroisoandrosterone has been shown by the conversion of the former into the latter by the male guinea pig (Miller and Dorfman, 1945). Whether this reaction is reversible so that dehydroisoandrosterone may be converted to and excreted in the urine as Δ^5-androstenediol-3(β),17(α) is being investigated at the present time. If the two steroids arise from a common intermediate or common intermediates, it would appear that dehydroisoandrosterone is further along the pathway since over 14 times more of the ketone was found in the urine than the diol.

The findings on the isolation of Δ^5-pregnenediol-3(β),20(α) in the two urines of the adrenal cortical cancer patient are of interest with respect to the great increase in concentration within an 18 week period. Twenty weeks before death of the patient some 0.33 mg. of this steroid was isolated per liter of urine, while 18 weeks later the concentration rose to 24.5 mg. per liter (Table 1). Although different methods of extraction were employed, it seems unlikely that the 74-

fold increase was due to difference in methods of extraction. Of further interest is the fact that the urine of patient B contained 0.18 mg. of Δ^5-pregnenediol-3(β),20(α) per liter which is of the same magnitude as that found for the first sample of the adrenal cortical cancer patient.

SUMMARY

The presence of Δ^5-pregnenediol-3(β),20(α) has been demonstrated in the urines from a woman suffering from an adrenal cortical cancer and a girl showing signs of virilism. Some twenty weeks before death the urine of the former patient yielded 0.33 mg. of Δ^5-pregnenediol-3(β),20(α) per liter; 18 weeks later 24.5 mg. per liter of this steroid were isolated. A yield of 0.18 mg. per liter of Δ^5-pregnenediol-3(β),20(α) was isolated from the urine of the girl showing signs of virilism.

The presence of Δ^5-androstenediol-3(β),17(α) was demonstrated in the urine of the adrenal cortical cancer patient. The yield was 48.0 mg. per liter in a urine sample collected 2 weeks before the patient's death.

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ENDOCRINE FUNCTION OF THE SPLEEN AND ITS PARTICIPATION IN THE PITUITARY-ADRENAL RESPONSE TO STRESS

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THE POSSIBILITY of the spleen functioning as an endocrine organ has been suspected for a long time. Extensive reviews of the problem by Lauda (1933) and Perla & Marmorston (1935) showed however that, in spite of a great deal of circumstantial evidence, there was no valid proof of an internal secretion of the spleen. The last ten years do not seem to have brought forward any decisive fact and it may be assumed that the results reported in this paper constitute the first experimental evidence that the spleen produces and can release into circulation a substance acting like a hormone. It is also shown that this function of the spleen is under the control of the pituitary and the adrenal cortex. The significance of the internal secretion of the spleen is not yet quite clear but all the data lead to the conclusion that it is produced in case of emergency and its main action is on protein metabolism.

METHODS

Guinea-pigs were used in most experiments. Where hypophysectomy or adrenalectomy was required, the experiments were performed on albino rats. These operations. as well as splenectomy, were performed under ether anaesthesia.

Bleeding time was measured on the ear; this was pricked with a needle (1 mm. diameter) and bleeding was recorded every five seconds by means of a blotting paper. Bleeding was considered to have stopped when no issue of blood or any fluid could be detected for 10 sec. The ear was not shaved and no antiseptic was applied before or after pricking. Measurements were done in unanaesthetized guinea-pigs but in rats light ether anaesthesia had to be given.

Blood samples for the measurement of antitryptic activity were collected by heart puncture. After clotting, the serum was separated and 0.025 cc. serum was incubated with 125 mg. gelatin and 5 mg.crude trypysin. Each sample was diluted to 5 cc. with phosphate buffer at pH 8. Both gelatin and trypsin solutions were strongly acid and were adjusted to pH 8 shortly bebefore use. Amino-N was determined before and after incubation for two hours at 38° C. Antitryptic activity was expressed in terms of the amount of trypsin inhibited by 0.025 cc. serum. This was calculated by comparing the

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increase of amino-N in the test and in control samples not containing serum.

Other techniques used were described in previous publications. Histamine release by blood cells was estimated with a method described by Gotzl & Dragstedt (1942) with slight modifications (Ungar, 1944a). Measured tissue damage was inflicted by a technique expressing the amount of trauma in terms of the energy spent by the traumatizing agent and measured in kg. m. (Ungar, 1943).

Results were tabulated as means of several (generally 8 or 16) measurements. Variation of the mean was expressed in terms of the standard error (S.E.). Significance of the differences between means was tested by the *t* test (Fisher & Yates, 1943) and the differences were considered significant when the chance was less than 1/20 that the means represented two samples from the same population.

RESULTS

a) Endocrine response to tissue injury, haemorrhage, starvation and drug actions.

It was reported previously that the blood of traumatized animals contains a substance probably identical with the corticotrophic hormone of the pituitary (Ungar, 1944a). The identification was based on the fact that the substance is not released in hypophysectomized animals and that its effect is inhibited by adrenalectomy. It was then assumed that tissue injury stimulates the secretion of corticotrophic hormone by the pituitary and thus induces secretion in the adrenal cortex. The test used in this work was the histamine release from blood cells *in vitro* which is inhibited by the serum of traumatized animals, corticotrophic hormone and whole cortical extract.

It was observed later that all the stimuli which inhibited histamine release also reduced bleeding time and increased capillary resistance. The reduction of bleeding time was selected as the easiest and most reliable test of the substances involved in the endocrine reaction to trauma and other conditions of stress. In 240 normal guinea-pigs the mean bleeding time was 131 ± 2 sec. There was no significant difference according to sex or age. In 34 normal rats, under light ether anaesthesia, the mean bleeding time was 238 ± 12 sec. In both species, the maximum reduction of the mean bleeding time is about 40 to 45%. Within this range, the reduction is quantitative and is linearly related to the log. dose.

In some experiments, concomitantly with bleeding time, capillary resistance was measured with the negative pressure method. On the whole, the results were less consistent than with the bleeding time and the variations did not follow any standard distribution. A pharmacological study of bleeding time and capillary resistance as methods of assay will be published elsewhere. It can be stated here that, for the substances considered in this paper, capillary resistance increased whenever bleeding time was reduced.

In a first series of experiments the effect of various stimuli was investigated in normal, hypophysectomized and adrenalectomized rats.

Table I shows that in normal rats the bleeding time is significantly reduced 24 hours after tissue injury. Serum from traumatized animals when injected subcutaneously into non traumatized rats caused a maximal reduction of bleeding time at the dose of 10^{-2} cc. Subcutaneous injections of corticotrophic hormone and adreno-cortical extract have the same effect in suitable doses. In hypophysectomized rats trauma is ineffective but in all other cases significant reductions of bleeding time were observed. In adrenalectomized rats, trauma, serum from traumatized animals and corticotrophic hormone had no effect but the adreno-cortical extract caused a reduction of bleeding time.

TABLE I. EFFECT OF VARIOUS STIMULI ON MEAN BLEEDING TIME IN NORMAL, HYPOPHYSECTOMIZED, ADRENALECTOMIZED AND SPLENECTOMIZED RATS

Treatment	Normal rats			Hypophysect.			Adrenalect.			Splenectomized		
	B.T.	±S.E.	N.	B.T.	±S.E.	N.	B.T.	±S.E.	N.	B.T.	±S.E.	N.
Control	238	12	34	246	20	8	253	25	22	244	27	8
Trauma, 0.97 kg.m. 24 h. after	*152*	23	8	260	28	8	265	21	8	257	18	8
Serum from traumatized g.-p., 10^{-2} cc.[1]	*150*	14	8	*157*	18	8	250	28	8	247	27	8
Corticotr. hormone 10^{-3} mg.[1]	*152*	24	8	*156*	20	8	303	22	8	288	28	8
Whole cort. extr., 1 g.[1]	*147*	23	8	*150*	18	8	*159*	22	8	234	27	8
Spleen extr. 0.5 mg.[1]	*156*	25	8	*162*	26	8	*148*	24	8	*160*	16	8

B.T. = mean bleeding time (sec.); S.E. = standard error of the mean; N = number of experiments. Figures in italics are significantly lower than the control.

[1] Injections were given subcutaneously and the doses expressed per kg. body weight. The bleeding time was measured 1 h. after injection.

The data summarized in Table I give further support to the assumptions that the pituitary is the first organ to react to tissue damage and that the substance detected in the blood of traumatized animals is corticotrophic hormone which stimulates the adrenal cortex and thus causes the release of a cortical hormone, Further investigation showed that the pituitary-adrenal response was not the whole reaction. As shown in Table I, in splenectomized rats the bleeding time was unaffected by all stimuli except by the injection of spleen extract. It was therefore reasonable to assume that the spleen participated in the endocrine response to tissue injury (Ungar, 1944c). A detailed investigation into the nature of the part played by the spleen was then conducted, using guinea-pigs.

Table II shows that after muscular injury the bleeding time is significantly reduced from the sixth hour until the eighth day. The post-traumatic reduction of bleeding time was first described by Fleisch & Posternak (1943) and confirmed by Ungar (1944b) and Scarborough (1944). In splenectomized animals, trauma not only fails to cause shortening of the bleeding time but induces a definite increase. Blood loss amounting to 1% body weight, drawn from the heart, has no

TABLE II. ACTION OF TISSUE INJURY, HAEMORRHAGE AND STARVATION ON MEAN BLEEDING TIME IN NORMAL AND SPLENECTOMIZED GUINEA-PIGS

	Interval	Normal			Splenectomized		
		B.T.	±S.E.	N.	B.T.	±S.E.	N.
Control	—	131	2	240	129	5	48
Trauma, 0.97 kg.m.	1 h.	139	11	16	135	14	8
	4 h.	134	13	16		—	
	6 h.	87	9	16	143	14	8
	1 day	83	8	16		—	
	2 days	75	8	16	175	24	8
	3 days	78	5	16		—	
	4 days	81	7	16	152	18	8
	5 days	92	7	16		—	
	8 days	127	8	16	132	20	6
Haemorrhage, 1% body wt.	1 h.	133	13	8		—	
	4 h.	129	20	8		—	
	6 h.	131	15	8		—	
Haemorrhage, 2.5% body wt.	1 h.	104	24	8		—	
	4 h.	88	18	8	134	15	8
	6 h.	131	15	8		—	
Starvation	1 day	130	17	8		—	
	3 days	132	19	8		—	
	4 days	97	14	8	129	13	8
	5 days	97	14	8		—	
	6 days	82	16	8	129	15	8
Start feeding	1 day	112	13	8		—	
	2 days	125	17	7		—	

B.T. = mean bleeding time (sec.); S.E. = standard error of the mean; N = number of experiments.

effect on bleeding time but with the loss of 2.5% the bleeding time is already shortened 1 h. after, reaches its maximum at 4 h. and returns to normal 6 h. after. In guinea-pigs submitted to starvation (supplied with water) bleeding time is shortened from the 4th day and reaches its minimum by the 6th day. If the animals are then fed, the bleeding

TABLE III. EFFECT OF SERUM FROM TRAUMATIZED AND NORMAL GUINEA-PIGS ON MEAN BLEEDING TIME IN NORMAL AND SPLENECTOMIZED GUINEA-PIGS

	Dose	Normal			Splenectomized		
	cc/kg	B.T.	±S.E.	N.	B.T.	±S.E.	N.
Control	—	131	2	240	129	5	48
Serum collected 24 h. after trauma	10^{-4}	114	17	8		—	
	10^{-3}	94	13	8		—	
	10^{-2}	80	14	8	157	17	8
	10^{-1}	106	17	8		—	
	3×10^{-1}	112	15	8		—	
	2	128	18	8	212	32	8
Normal serum	10^{-4}	131	11	8		—	
	10^{-3}	127	12	8		—	
	10^{-2}	139	15	8		—	
	10^{-1}	155	15	8	162	20	8
	1	167	26	8		—	

Serum was given by subcutaneous injection and bleeding time was measured 1 h. after injection. B.T. = mean bleeding time; S.E. = standard error of the mean; N = number of experiments.

time returns to normal in 48 hours. In splenectomized animals the bleeding time remains unaffected by these stimuli. It is to be noted that splenectomy by itself does not alter the bleeding time.

In a further series of experiments the effect of the serum from traumatized animals was considered. Table III shows that 10^{-4} cc.

TABLE IV. EFFECT OF PITUITARY CORTICOTROPHIC HORMONE, ADRENO-CORTICAL EXTRACTS AND DESOXYCORTICOSTERONE ACETATE ON MEAN BLEEDING TIME IN NORMAL AND SPLENECTOMIZED GUINEA-PIGS

	Dose/kg.	Normal B.T.	±S.E.	N.	Splenectomized B.T.	±S.E.	N.
Control	—	131	2	240	129	5	48
Corticotrophic hormone	2×10^{-5} mg.	103	15	8		—	
	10^{-4} mg.	93	13	8		—	
	3×10^{-4} mg.	82	9	8		—	
	10^{-3} mg.	73	6	16	129	17	8
	10^{-2} mg.	84	12	8		—	
	10^{-1} mg.	142	12	8		—	
	1 mg.	199	22	8	199	30	8
"Whole cortical extract" of Kendall[1]	30 mg.	120	12	8		—	
	100 mg.	106	16	8		—	
	1 g.	84	7	12		—	
	10 g.	72	6	8	131	12	8
	100 g.	77	10	8	127	13	8
Guinea-pig adrenal extract in saline[1]	0.5 mg.	116	23	8		—	
	5 mg.	75	10	8	134	16	8
Desoxycorticosterone acetate	1 mg.	129	13	8		—	
	10 mg.	125	8	8		—	

Bleeding time was measured 1 h. after subcutaneous injection.

[1] Doses are expressed in weight of fresh tissue.

B.T. = mean bleeding time (sec.); S.E. = standard error of the mean; N = number of experiments.

serum per kg. body weight given in subcutaneous injection reduces bleeding time and the maximum effect is reached with 10^{-2} cc. This effect diminishes progressively with doses above this level. This can probably be explained by the fact shown in the table that normal guinea-pig serum increases bleeding time when the dose injected is greater than 10^{-2} cc. In serum collected from traumatized animals both the reducing and increasing substances are present and, according to the dose, one or the other is observed. In splenectomized animals only the increasing effect is produced suggesting that the substance responsible for the reduction of bleeding time acts through the spleen.

The action of the pituitary corticotrophic hormone is shown in Table IV. A fairly impure preparation[2] given by subcutaneous injection reduces bleeding time in the dose as small as 2×10^{-5} mg./kg. The maximum effect is reached with 10^{-3} mg. and further increase of dose results in a reversal of the effect until with 1 mg. the bleeding

[2] I am indebted to Dr. A. N. Macbeth of Organon Laboratories Ltd. for generous supply of corticotrophic hormone.

time is significantly higher than the normal. It was shown in adrenalectomized rats that the increase of bleeding time is not mediated by the suprarenals. Table IV shows that it is not affected by splenectomy while the shortening effect is entirely inhibited by it. It is therefore likely that the bleeding time increasing effect is due to an impurity.

Table IV also shows the effect of a cortical preparation, labelled "Whole cortical extract."[3] Subcutaneous injection of the dose cor-

TABLE V. EFFECTS OF VARIOUS DRUGS ON MEAN BLEEDING TIME IN NORMAL AND SPLENECTOMIZED GUINEA PIGS

Drug	Dose/kg.	Interval	Normal			Splenectomized		
			B.T.	±S.E.	N.	B.T.	±S.E.	N.
Control	—	—	131	2	240	129	5	48
Peptone	100 mg.	1 h.	143	13	16	172	12	8
	100 mg.	5 h.	71	5	16	148	13	8
	100 mg.	24 h.	132	9	16		—	
Nupercaine HCl	4 mg.	1 h.	158	15	16	253	23	8
	4 mg.	5 h.	82	7	16	132	11	8
	4 mg.	24 h.	88	6	16		—	
	4 mg.	48 h.	151	11	16		—	
Choline chloride	1 mg.	1 h.	113	13	8		—	
	10 mg.	1 h.	97	16	8		—	
	40 mg.	1 h.	82	11	8	129	11	8
Ascorbic acid	50 mg.	5 h.	110	13	8		—	
	100 mg.	5 h.	87	8	16		—	
	200 mg.	5 h.	79	8	8	81	17	8
"Vitamin P," Hesperidin methyl chalcone	0.75 mg.	4 h.	131	17	8		—	
	2 mg.	4 h.	101	15	8		—	
	5 mg.	4 h.	76	13	8	76	17	8
"Vitamin P," Catechin	10^{-4} mg.	3 h.	99	12	8		—	
	4×10^{-2} mg.	3 h.	75	9	8		—	

All substances were injected subcutaneously, with the exception of peptone given by the peritoneal route.

B.T. = mean bleeding time (sec.); S.E. = standard error of the mean; N = number of experiments.

responding to 100 mg. fresh tissue shortens the bleeding time and the maximum effect is reached with 10 g. No reversal of the effect is observed with higher doses. A crude guinea-pig adrenal extract is about a thousand times more active which suggests that the ordinary methods of extraction eliminate or destroy most of the substance responsible for the bleeding time reducing effect. Desoxycorticosterone acetate in doses of 1 and 10 mg./kg. is entirely inactive. All the measurements summarized in Table IV were done 1 h. after injection. Desoxycorticosterone acetate was however also tested at longer intervals up to 48 h. but always with negative results. Table IV shows that in splenectomized guinea-pigs none of the adreno-cortical preparations had any effect on bleeding time.

[3] I am grateful to Dr. S. Zuckerman for supplying me with the "Whole cortical extract" sent to him by Dr. E. C. Kendall.

In the course of an inquiry into changes of bleeding time brought about by drugs, quite a number of these were found capable of increasing bleeding time while only comparatively few drugs had a reducing effect. Full results of the pharmacological investigations will be published elsewhere. As Table V shows, the first group of the drugs which reduce bleeding time (peptone, nupercaine, choline) is inactive in splenectomized animals or can even increase bleeding time. The second group, comprising ascorbic acid and "Vitamin P" is still active after splenectomy. These facts support the opinion already expressed

TABLE VI. EFFECT OF CRUDE SPLEEN EXTRACT AND CRYSTALLINE "SPLENIN" ON MEAN BLEEDING TIME IN NORMAL AND SPLENECTOMIZED GUINEA-PIGS

	Dose mg/kg.	Normal			Splenectomized		
		B.T.	±S.E.	N.	B.T.	±S.E.	N.
Control	—	131	2	240	129	5	48
Spleen extract in saline[1]	5×10^{-2}	104	15	8		—	
	10^{-1}	98	14	8		—	
	3×10^{-1}	79	14	8	73	12	8
	1	98	10	8		—	
	10	132	12	8		—	
	100	176	23	8	186	19	8
Crystalline splenin	2×10^{-7}	109	17	8		—	
	3×10^{-7}		—		102	16	8
	10^{-6}	88	13	8		—	
	2×10^{-6}	84	9	8		—	
	10^{-5}	78	7	8		—	
	10^{-4}	76	6	8		—	

Bleeding time was measured 1 h. after subcutaneous injection.
[1] Dose expressed in weight of fresh tissue.
B.T. = mean bleeding time (sec.); S.E. = standard error of the mean; N = number of experiments.

(Ungar, 1944a) that peptone and local anaesthetics stimulate the release of corticotrophic hormone while ascorbic acid exerts the same effect by some direct mechanism. There is a third group of substances which reduce bleeding time, represented by pituitrin and, in special conditions, by adrenaline, but they act by a different mechanism and their effect bears no relation to the present problem.

b) Presence of an active substance in the spleen

The results just reported suggested that under the influence of certain stimuli a reaction took place involving the pituitary, the adrenal cortex and the spleen. The question then was whether the spleen played its part by releasing an active substance or by some other mechanism. A crude extract of guinea-pig spleen in saline was prepared and injected subcutaneously in order to study its effect on the bleeding time of guinea-pigs. Table VI shows that bleeding time is reduced by a dose equivalent to 5×10^{-2} mg. fresh tissue and the maximum effect is reached by 3×10^{-1} mg. If the dose is further increased, the effect is reversed and with 100 mg. the bleeding time is significantly longer than the normal.

The active substances responsible for the two opposing effects were subsequently separated. The bleeding time increasing substance is probably identical with the "Thrombocytopen" isolated from spleen by Troland & Lee (1938). There is no evidence that this substance is released in the conditions considered in this paper. The increase of bleeding time observed after certain stimuli (Tables II, III, IV, V and VI) is not mediated by the spleen. It is however, possible that this substance plays a part in certain pathological conditions which are now being investigated.

Isolation and identification of the bleeding time reducing substance has been carried out with the collaboration of Dr. R. H. Martin. Details will be published as soon as the structure is definitely established. The substance which will be referred to provisionally as "Splenin" is a crystalline solid of rather low molecular weight and low melting point. It is soluble in water, ether and chloroform, insoluble in acetone and concentrated methyl alcohol. It is stable at ordinary temperature and in neutral or slightly alkaline solution but is quickly destroyed at 100° C. and in acid solution.

Remarkably low concentrations of splenin are sufficient to shorten bleeding time. Table VI shows a reduction after subcutaneous injection of 3×10^{-7} mg./kg. and the maximum effect is reached with 10^{-6} mg. There is no reversal of the effect with higher doses. The action of splenin is not inhibited by splenectomy. Splenin also increases capillary resistance and inhibits histamine release by blood cells.

In order to decide whether splenin was produced by the spleen only, the effect on bleeding time of extracts from a number of organs was studied. The organs were removed from guinea-pigs killed by bleeding and extracted with saline. Amounts corresponding to 50 and 0.5 mg. fresh tissue were injected and bleeding time was measured 1 h. after. Extracts from the following organs had no effect on bleeding time: liver, kidney, lung, brain, stomach, duodenum, skeletal muscle, skin, thymus, lymph gland, pancreas, thyroid, testes and blood cells. As was to be expected, positive results were obtained with pituitary and adrenal extracts but their effect was inhibited by splenectomy. The results indicate that the organs investigated contained, if any, less than 10^{-6} mg. splenin per g. of fresh tissue. Spleen from various species (guinea-pig, rat, mouse, rabbit, sheep, ox) contains about 10^{-3} mg. per g. fresh tissue.

Normal serum and urine probably contain small amounts of splenin. Ether extracts of rabbit serum and human urine were prepared and tested for their action on bleeding time in splenectomized guinea-pigs. Normal rabbit serum was found to contain about 6×10^{-7} mg. splenin per cc. After trauma, this goes up to 4×10^{-6} mg. In splenectomized rabbits, serum contains less than 10^{-7} mg. and trauma fails to bring about an increase. Splenin content of normal human urine was estimated at about 10^{-8} mg./cc.

These results are based on the assumption that the bleeding time

reducing substance present in ether extracts of serum or urine is splenin. This is however not yet definitely established. The reliability of the method is now being investigated in order to start a systematic study of the metabolism of splenin.

c) *Mode of action of splenin.*

Splenin was found to have the following effects: (1) reduction of bleeding time, (2) increase of capillary resistance and (3) inhibition of histamine release from blood cells. Considering the possible relation-

TABLE VII. EFFECT OF VARIOUS STIMULI ON SERUM ANTITRYPSIN IN GUINEA-PIGS

	Interval[1]	Amount of trypsin inhibited (mg.)	±S.E.
Control	—	1.6	0.1
Injection of splenin, 10^{-4} mg.	1 h.	3.5	0.3
Trauma, 0.97 kg.m.	24 h.	2.3	0.2
Splenectomy	8 days	0.9	0.1
Trauma after splenectomy	24 h.	0.7	0.1

Each figure represents the mean of the results of 8 experiments.
[1] Interval between the stimulus and the collecting of blood samples.

ship between these actions, which will be discussed below, it seemed likely that they might depend on some common mechanism connected with protein metabolism. It was therefore decided to investigate whether splenin had anything to do with the mechanism which prevents the breakdown of proteins in blood by trypsin and other proteases.

In the first series of experiments antitryptic action of serum was compared in normal guinea-pigs and in animals submitted to subcutaneous injections of splenin, trauma and splenectomy. Table VII shows that after injection of 10^{-4} mg. splenin the antitryptic action of serum is very significantly increased. After trauma there is also an increase, smaller but still significant. In splenectomized guinea-pigs the antitryptic action goes down and, when these animals are traumatized, no increase is observed.

These results suggested that the spleen contributed in some way to the antitryptic action of serum and that this action was raised whenever splenin was released. Another series of experiments was devised, to investigate the mechanism by which splenin increases the antitryptic action. It has been known for a long time that antitryptic action is suppressed when serum is extracted with ether and that it cannot be recovered from the ether extract. Table VIII shows that this was confirmed: the aqueous fraction has only a very slight antitryptic power and the ether fraction evaporated and redissolved in water, is practically inactive. However, if both fractions are put together again, the antitryptic action of the original serum is restored. This suggests that the inhibition of trypsin requires the cooperation of two substances, one soluble in water and another soluble in ether. The water soluble substance is probably the antitrypsin, crystallized

by Kunitz & Northrop (1936), which, when present in small amounts, may require an activator, a "co-antitrypsin."

The question then was whether splenin could not play the part of this activator. Table VIII shows that by itself splenin has no antitryptic power but it is capable of restoring the activity of the water soluble fraction. The antitryptic action of the mixture increases with the concentration of splenin. It remains still to be decided whether

TABLE VIII. ANTITRYPTIC ACTION OF FRACTIONS OF GUINEA-PIG SERUM AND THE EFFECT OF SPLENIN

	Amount of trypsin inhibited (mg.)	±S.E.
Total serum	1.55	0.07
Aqueous fraction	0.35	0.05
Ether fraction	0.15	0.01
Aqueous fraction + Ether fraction	1.75	0.11
Aqueous fraction + Splenin 10^{-7} mg.	1.10	0.09
Aqueous fraction + Splenin 10^{-6} mg.	2.25	0.10
Aqueous fraction + Splenin 10^{-5} mg.	3.35	0.25
Splenin 10^{-6} mg.	0	—

Each figure represents the mean of the results of 10 experiments.

splenin is the normal activator of antitrypsin or only acts as such in case of emergency.

DISCUSSION

The results just described lead to the conclusion that certain stimuli elicit an endocrine response involving the pituitary, the adrenal cortex and the spleen. This response is probably the same phenomenon as the "alarm reaction" of Selye (1937) who described the adrenal hypertrophy induced by the application of noxious stimuli. The problem of adrenal hypertrophy was reviewed more recently by Tepperman, Engel & Long (1943) who showed that the primary stimulus is probably some change in protein metabolism acting on the pituitary.

Selye showed that the alarm reaction has the significance of an adaptation, i.e. an increased resistance which is not limited to the originating stimulus but extends to a group of similar stimuli. Relations between this reaction and non-specific resistance, skeptophylaxis and peptone refractoriness were discussed elsewhere (Selye, 1937; Ungar, 1944a). As far as the adaptation is concerned, it is not yet clear whether the spleen plays any part in it. Splenectomized animals behave in a perfectly normal manner, and in some cases at least, do not exhibit the decreased resistance which is observed in adrenalectomized animals (Perla & Marmorston, 1933; Perla, 1934). There is however, one striking difference between normal and splenectomized animals which has been extensively investigated. It is known that many laboratory stocks of rats are infected with *Bartonella muris*.

The disease remains latent in normal animals but its symptoms appear as soon as the spleen is removed. Lauda & Flaum (1930) have shown in parabiotic rats that the protective action of the spleen is effected through some diffusible substance. Perla & Marmorston-Gottesman (1932) obtained some results in protecting splenectomized rats against Bartonella anaemia by injecting an ether extract of spleen. Preliminary experiments on the effect of tissue injury in normal and splenectomized animals show a significantly higher mortality in the latter group.

Reduction of the bleeding time by spleen extracts was first observed by Landau in 1904. The importance of this action is to supply a sensitive, reliable and easy test for the study of splenin. It is likely that the failure of earlier workers to detect and isolate the active substance from spleen can be explained by the lack of a convenient test. Action on bleeding time is however probably only a secondary effect. In 1941, Macfarlane proposed a theory of bleeding time based on the assumption that tissue injury releases vasodilator substances. The duration of capillary bleeding would depend on the amount of these substances released. Macfarlane's theory is supported by the fact that stimuli which reduce bleeding time also inhibit the release of histamine. On the other hand it has been assumed that histamine is released as a result of enzymic proteolysis (Feldberg, 1941). Histamine and probably other vasodilator substances are loosely combined with proteins and can be set free by trypsin (Rocha e Silva, 1944). The effect of trypsin depends however on the amount of antitrypsin and its activator present. The results reported above show a correlation between antitryptic action, inhibition of histamine release and reduction of bleeding time. It is therefore reasonable to assume that whenever splenin is released, antitryptic power is increased, liberation of vasodilator protein breakdown products is inhibited and bleeding time is reduced.

What is the bearing of the observations reported in this paper on the post-traumatic nitrogen catabolism described by Cuthbertson (1929) and confirmed recently by numerous investigators? Attempt has been made to explain the period of negative nitrogen balance which follows tissue injury by an endocrine reaction involving the pituitary and the adrenal cortex. According to the latest hypothesis (Albright, 1943), the metabolic changes would be due to the release of an anti-anabolic hormone. The part played by the spleen in this reaction is not yet quite clear. As a tentative explanation, it can be assumed that splenin is part of an antienzyme system which inhibits not only proteolysis but also the building up of new proteins and would thus act by adjusting protein metabolism at a lower level.

Can it be legitimately claimed, in the light of the facts reported in this paper, that the spleen is an endocrine organ? The principal arguments are: (1) demonstration of a highly active substance produced by the spleen alone, (2) liberation of the active substance

into the blood stream by appropriate stimuli and (3) failure to detect the active substance in the blood of splenectomized animals submitted to the same stimuli. Some more evidence is required concerning the significance of the spleen response to various stimuli in order to decide whether splenin can be considered as an important hormone.

SUMMARY

In the course of a study of the pituitary-adrenal reaction to stress, this response was found to be inhibited by splenectomy. It was also observed that spleen extracts contain an active substance which can reproduce the reaction to the original stimulus. This active substance was isolated and obtained in pure, crystalline form. It is probably part of the anti-enzyme system which protects proteins against trypsin. The spleen, as an endocrine organ, may therefore play a part in the control of protein metabolism and its adjustment to conditions of stress.

The significance of the pituitary-adrenal-spleen response and its relation to the alarm reaction and the post-traumatic nitrogen catabolism were discussed.

ACKNOWLEDGEMENT

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A COMPARISON OF ADRENAL STEROID DIABETES AND PANCREATIC DIABETES IN THE RAT

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THE C-11-oxygenated adrenal cortical steriods have the property of stimulating gluconeogenesis from protein (Long, Katzin and Fry, 1940) and probably of inhibiting the utilization of carbohydrate (Ingle and Thorn, 1941). How are these two effects related? Do they represent independent actions of the cortical hormones or is one effect primary to the other? Ingle (1942) and Drury (1942) have suggested that the inhibition of carbohydrate utilization may cause the increase in gluconeogenesis since an increase in protein catabolism also occurs during pancreatic diabetes, phlorhizin diabetes, starvation etc. If this hypothesis is correct it should be possible to modify the extent of nitrogen loss during adrenal steroid diabetes by treatment with insulin.

The present study was intended to test the above hypothesis by comparing the effects of insulin on the nitrogen, electrolyte and carbohydrate balance of rats having pancreatic diabetes with the effects of insulin in similar animals having adrenal steroid diabetes induced according to Ingle (1941). The results indicate that the effect of the C-11-17-oxygenated cortical steroids in stimulating protein catabolism is not prevented or significantly diminished by insulin. Adrenal steroid diabetes but not pancreatic diabetes was highly resistant to control by insulin. Changes in body-weight and in the urinary excretion of nitrogen, chloride, sodium and potassium followed either the administration of cortical steroids or the withdrawal of insulin from the depancreatized animal.

METHODS

Male rats of the Sprague-Dawley strain were maintained on a stock diet of Purina Dog Chow until the beginning of the metabolism studies. Partial pancreatectomy was carried out at a body-weight of 200 to 225 grams in five rats. The technique of Ingle and Griffith (1942) was modified according to Richter and Schmidt (1939) so that the pancreas lying within the duodenal loop was removed by suction through a fine pipette. All of the pancreas was removed except approximately half of the pancreatic tissue lying between the bile duct and the duodenum. These severely diabetic rats were treated

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with 20 units of regular insulin (Lilly) in two divided doses per day per rat until the beginning of the metabolism studies. When the depancreatized animals and their unoperated controls reached weights of 300 to 325 grams they were placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:00 AM) and late afternoon (4:30 to 5:00 PM). The technique of force-feeding and the high carbohydrate diet used (Table 1) were modifications of those described by Reinecke, Ball and Samuels (1939). During the period of adaptation to force-feeding the diet was administered in small amounts to prevent the development of "food shock." The animals were brought to a full feeding on the fifth day. Each rat received 26 cc. of diet per day which contained approximately 15 grams of available carbohydrate.

The injections of insulin to the rats were made subcutaneously immediately following each feeding. Regular insulin and protamine zinc insulin

TABLE 1

Constituent	grams
Cellu Flour (Chicago Dietetic Supply)	120
Osborne and Mendel salt mixture	40
Wheat germ oil	10
Mazola Oil	10
Cod liver oil	10
Dried yeast (Pabst)	100
Egg albumin (Merck)	160
Starch	500
Dextrin	250
Sucrose	250
Water to make total volume of	2000 cc.

(Lilly) were used. The adrenal cortical steroids were prepared as fine suspensions in sesame oil and were administered by subcutaneous injection immediately following each feeding. The volume of oil was constant at one-half cc. per injection

Twenty-four-hour samples of urine were collected at the same hour each day and preserved with thymol with added citric acid (1 gram per sample) to insure the acidity of the samples for nitrogen analysis. The following methods of analysis were used: Blood glucose, Miller and Van Slyke (1936); urinary glucose, Benedict (1911); urinary chloride, Schales and Schales (1941); urinary sodium, Butler and Tuthill (1931) and urinary potassium, Consolazio and Talbott (1938). The determination of non-protein nitrogen was by the micro-Kjeldahl procedure as follows: Proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure. The organic matter was oxidized by sulfuric acid and hydrogen peroxide. The ammonia was distilled off into a standard acid solution and titrated with standard base.

EXPERIMENTS AND RESULTS

Following control periods of ten days, five unoperated rats were treated with the C-11-17-oxygenated adrenal steroids and insulin (Figures 1 to 5) and in parallel experiments five depancreatized rats were observed during periods of insulin treatment and withdrawal (Figures 6 to 10).

Urinary glucose. All of the unoperated rats were free from glycosuria during the control periods. The administration of 5.0 mgm. daily of 17-hydroxycorticosterone to rats 704 (Figure 1) and 809 (Figure 2) caused the development of glycosuria to a maximum of approximately eight grams per day in each animal. In rat 704 the administration of protamine zinc insulin in amounts up to 20 units daily caused a marked reduction in the amount of glucose excreted but the urine did not become sugar-free prior to the death of the animal. In rat 809, the administration of regular insulin in amounts

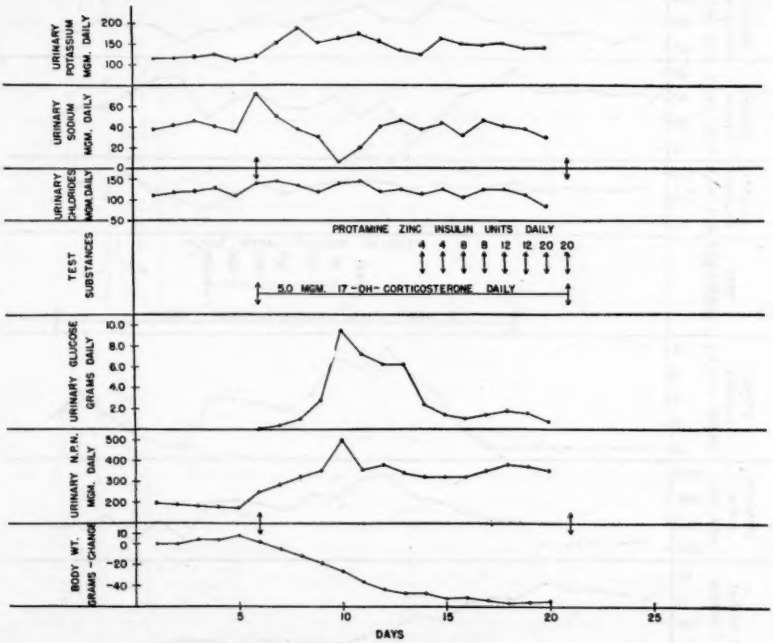

FIG. 1. Intact rat 704. In this and each of the other figures a change in the administration of a test substance is plotted on a vertical line with the data obtained at the end of the 24 hour period within which the change was made.

up to 1000 units daily caused a reduction in the amount of urinary glucose but did not abolish it. On the first day that insulin was withdrawn no glucose was excreted but on succeeding days the glycosuria reappeared and increased in severity during this phase of the experiment until the death of the rat.

Three rats were each treated with 5.0 mgm. per day of 17-hydroxy-11-dehydrocorticosterone. Rat 202 (Figure 3) remained free of glycosuria during the first seven days of steroid administration. It excreted a small amount of glucose on the first day of a five day period during which 8 units of protamine zinc insulin were given per

day and developed a marked glycosuria immediately following the withdrawal of insulin. When the administration of the steroid was stopped the glycosuria disappeared. Rat 601 (Figure 4) developed a mild glycosuria during the period of steroid administration which was abolished by the injection of protamine zinc insulin in amounts up to 8 units per day. The glycosuria reappeared when insulin was withdrawn but ceased when the injection of the steroid was stopped. Rat 502 (Figure 5) developed a severe glycosuria during the period

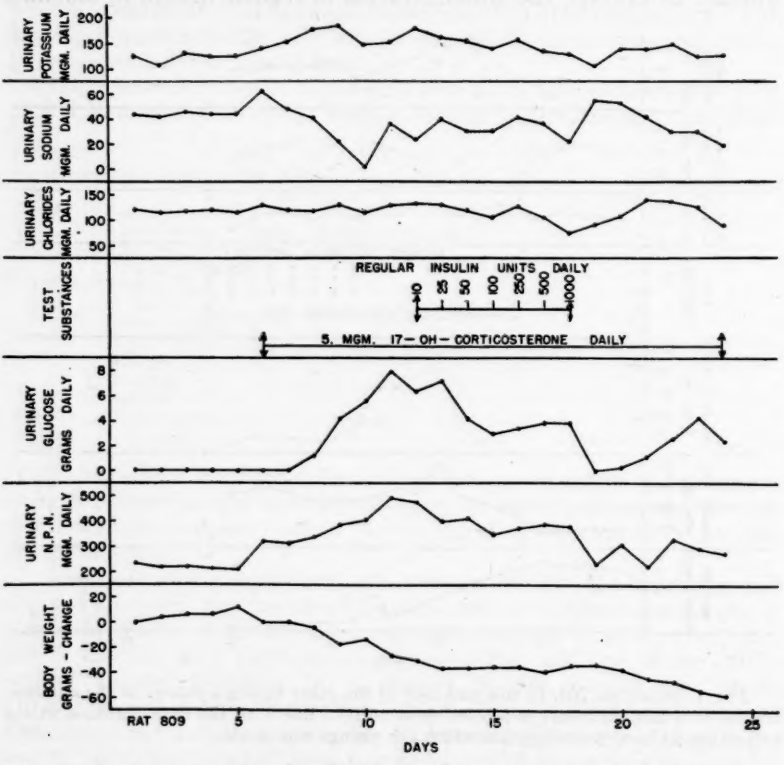

FIG. 2. Intact rat 809.

of steroid injection. The administration of protamine zinc insulin in amounts up to 100 units per day caused a marked but incomplete reduction in glycosuria. On the second day following the withdrawal of insulin the urine became free of glucose but on succeeding days the glycosuria reappeared and increased to a maximum level of 12.5 grams per day. At this point the steroid was withdrawn and the glycosuria disappeared.

The partially depancreatized rats were kept free of glycosuria by

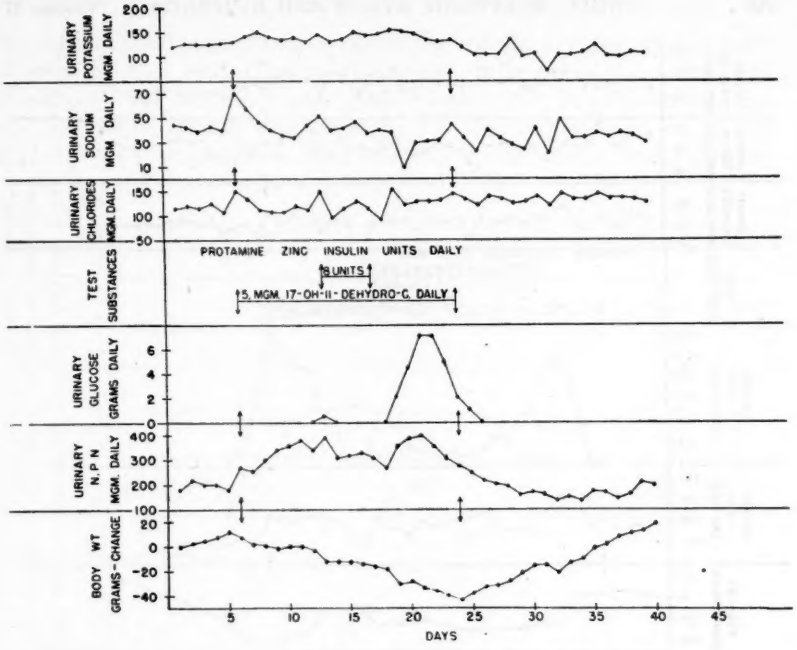

FIG. 3. Intact rat 202.

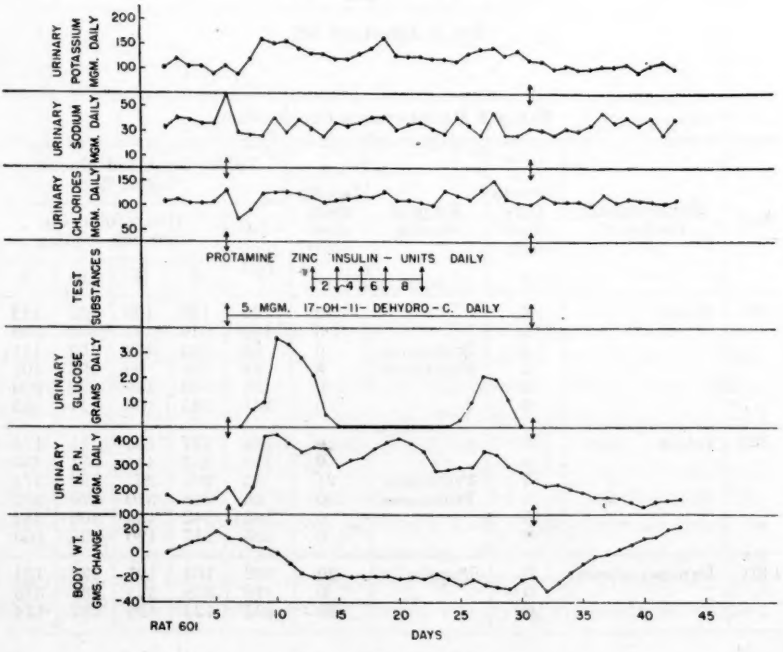

FIG. 4. Intact rat 601.

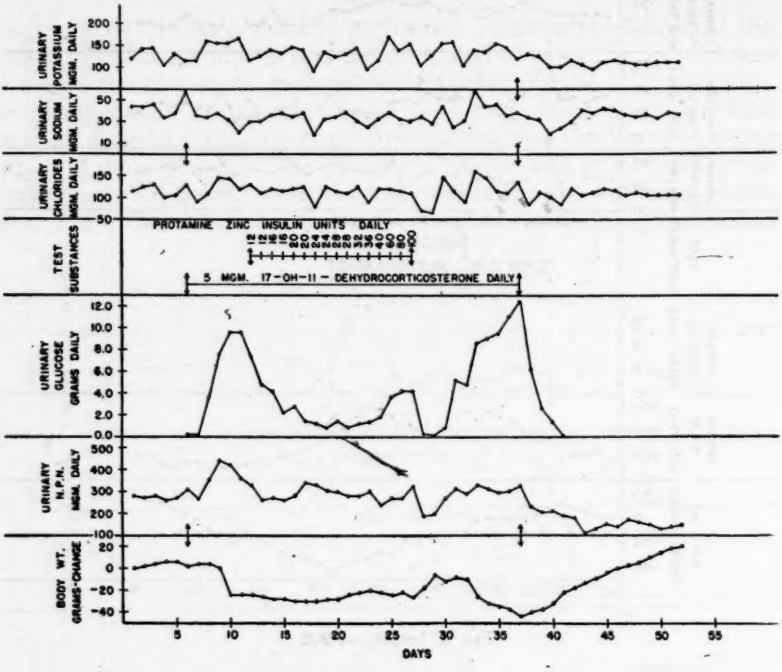

FIG. 5. Intact rat 502.

TABLE 2. VALUES FOR BLOOD GLUCOSE

Rat	Experimental condition	Steroid daily dose mgm.	Form of insulin	Insulin daily dose units	Values for blood glucose, mgm. %				
					16 hour fast	Hours following morning feeding			
						2	4	6	8
601	Intact	0		0	99	120	105	132	113
		5		0	102	249	341	360	310
		5	Protamine	6	86	120	112	102	111
		5	Protamine	8	84	108	99	108	105
		5		0	128	159	199	238	300
		0		0	101	105	106	111	103
502	Intact	0		0	114	117	135	111	124
		5		0	213	455	444	531	480
		5	Protamine	24	53	306	291	192	174
		5	Protamine	100	66	388	370	370	267
		5		0	185	342	489	409	382
		0		0	135	117	120	135	150
1401	Depancreatized	0	Regular	20	102	104	105	120	131
		0		0	349	338	345	334	315
		0		20	105	123	126	132	114

the administration of 20 units of regular insulin per day to rats 1006 (Figure 6) and 1401 (Figure 7) and 30 units per day to rats 805 (Figure 8), 905 (Figure 9), and 1403 (Figure 10). In each animal the withdrawal of insulin was followed by the development of severe glycosuria which persisted until treatment with insulin was renewed.

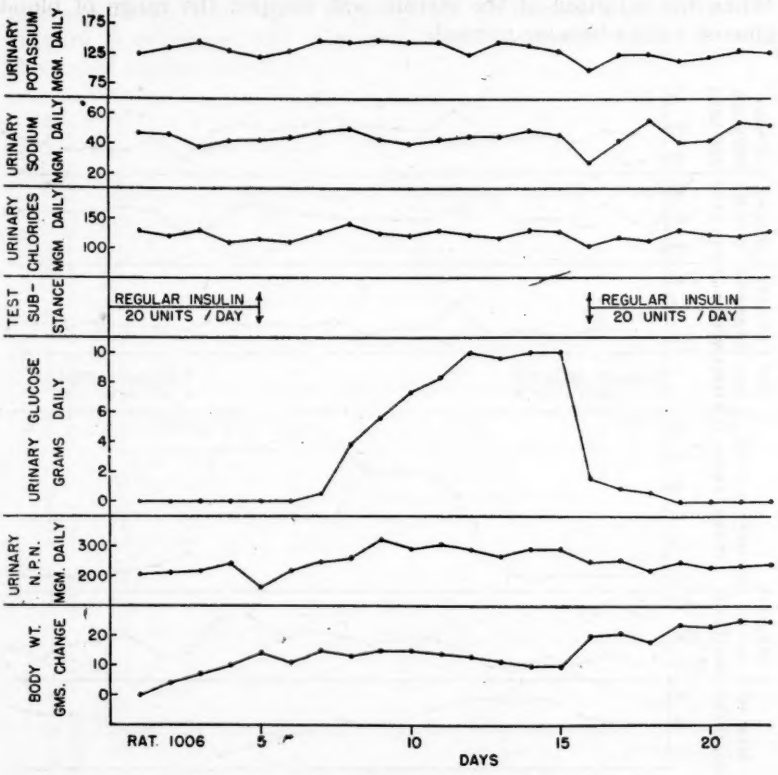

FIG. 6. Partially depancreatized rat 1006.

Blood glucose. Data which illustrate the changes in blood glucose observed in these experiments are summarized in Table 2. Once during each phase of the experiment samples of blood were taken from the tail just prior to the morning feeding (sixteen hour fast) and at two, four, six and eight hours following the morning feeding. All of the normal animals tolerated the high carbohydrate diet without the development of hyperglycemia prior to the injection of the steroids. During the injection of either 17-hydroxycorticosterone or of 17-hydroxy-11-dehydrocorticosterone all of the animals had hyperglycemia following feeding. The hyperglycemia of rats 202 and 601 was effectively controlled by insulin but hyperglycemia developed

following feeding in the remaining animals although insulin was given in large doses. In rats 502 and 809 which exhibited the greatest resistance to insulin the values for blood glucose were very low during fasting, a value of 24 mgm. per cent was recorded for rat 809, but they were rapidly increased to hyperglycemic levels following feeding. When the injection of the steroid was stopped the range of blood glucose values became normal.

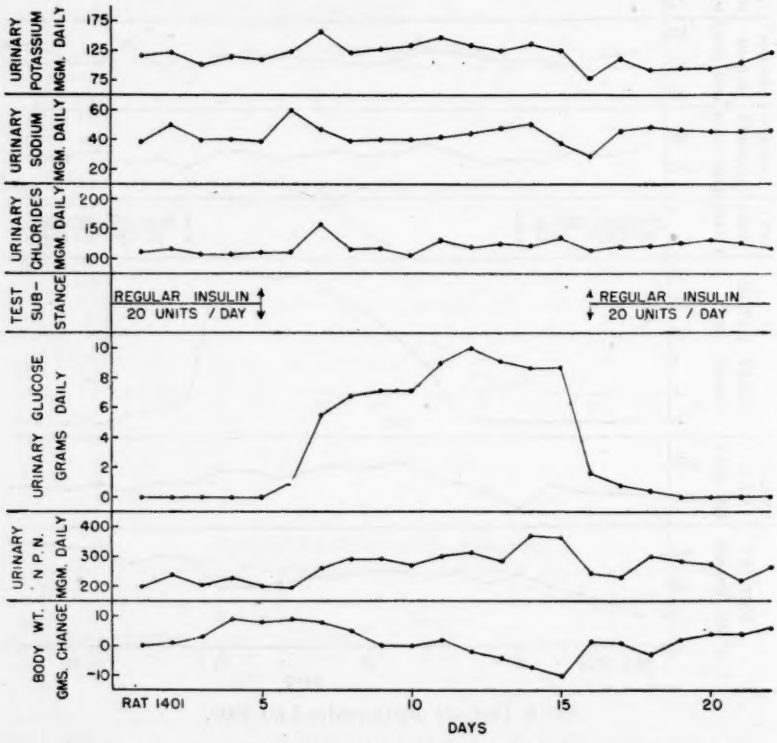

FIG. 7. Partially depancreatized rat 1401.

The depancreatized rats behaved in the expected manner during the presence and absence of insulin. Hyperglycemia developed when insulin was withdrawn and much lower, somewhat irregular values were observed during treatment with insulin.

Resistance to insulin. It was first planned to use only protamine zinc insulin in these experiments. In preliminary studies on depancreatized rats it was found that twice-daily doses of protamine zinc insulin (at times of feeding) which were sufficiently large to control the glycosuria would also cause periods of hypoglycemic shock. None

of the depancreatized rats were able to resist as much as 3 units per day of protamine zinc insulin without fatal hypoglycemia. These animals appeared to be less resistant to insulin over-dosage than normal rats under similar conditions (Ingle, Evans and Sheppard, 1944). Regular insulin was used successfully in these experiments although large doses were required for prolonged action. Rat 905 (Figure 9) developed the only case of insulin shock observed during the use of regular insulin.

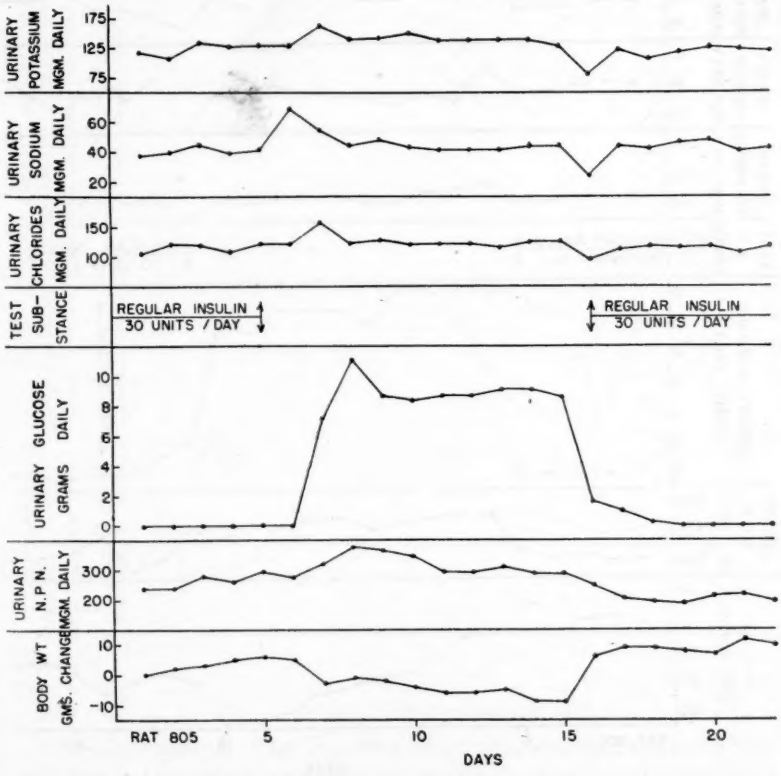

FIG. 8. Partially depancreatized rat 805.

All of the rats having adrenal steroid diabetes were abnormally resistant to insulin. In rats 502 and 809 the resistance was remarkably high although insulin had some effect upon the fasting level of blood glucose and upon urinary glucose.

Body-weight. The unoperated rats each gained weight during the pre-injection period and lost weight during the injection of the cortical steroids. The administration of insulin either retarded or

stopped the loss of weight but did not cause any significant gains. Upon withdrawal of insulin the animals lost weight until the injections of the cortical steroids were stopped whereupon rapid gains occurred.

The depancreatized rats gained weight during the administration of insulin but lost weight when insulin was withdrawn. With the exception of rat 905 which lost 34 grams, the depancreatized rats

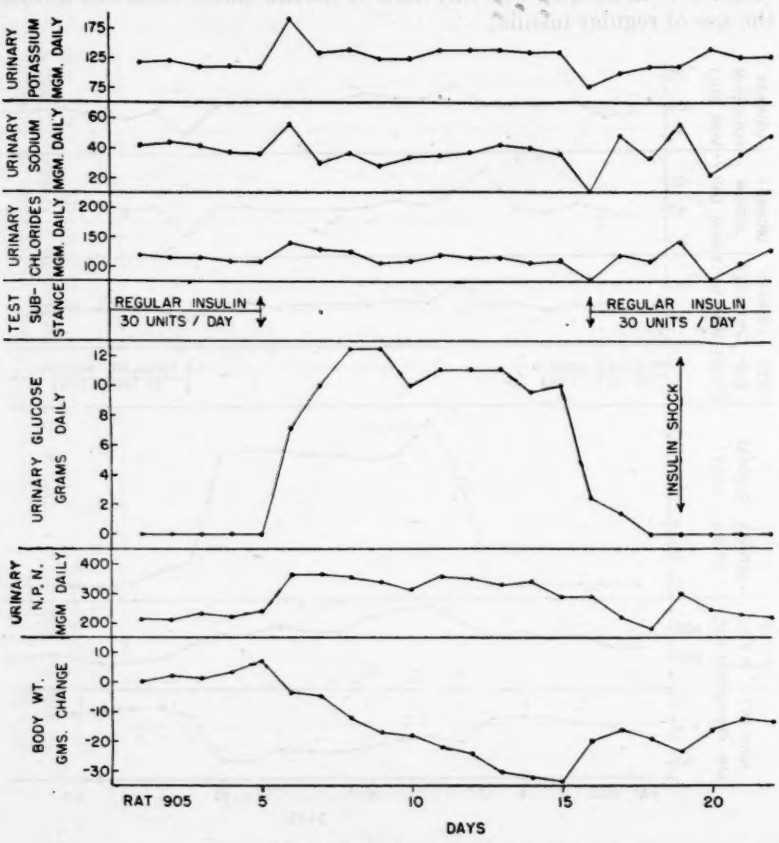

FIG. 9. Partially depancreatized rat 905.

lost much less weight than the animals treated with the cortical steroids.

Urinary non-protein nitrogen. In each of the unoperated rats the injection of the cortical steroid caused a marked increase in the urinary excretion of non-protein nitrogen. The peak of the rise was not sustained. There was no clear tendency for the injection of insulin to suppress the rise in urinary nitrogen. When insulin was withdrawn

from those rats which survived the nitrogen of the urine was not increased. When the injections of the steroids were stopped the nitrogen balance became strongly positive. Following the withdrawal of insulin from the depancreatized rats there was a rise in urinary nitrogen but the increase was less than in the animals which were injected with the adrenal steroids.

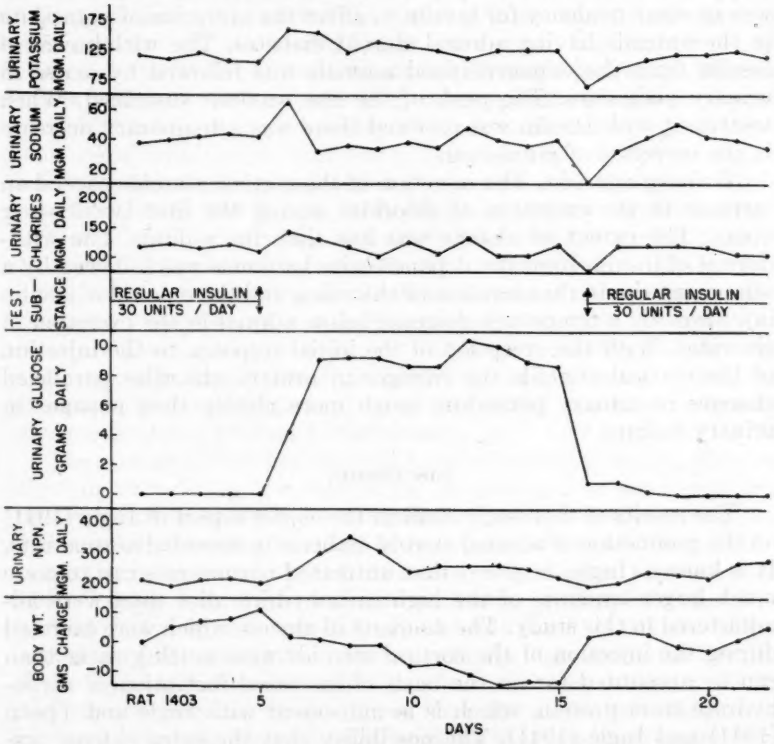

FIG. 10. Partially depancreatized rat 1403.

Urinary sodium. The injection of cortical steroids caused a sharp increase in the excretion of sodium during the first twenty-four hours. This temporary increase was followed within a few days by a period of increased retention. There were no consistent changes related to the period of insulin injection and withdrawal or to the withdrawal of the cortical steroids. During the first twenty-four hours following the withdrawal of insulin from the depancreatized rats there was a sharp increase in the excretion of sodium in four animals and a slight increase in one. This temporary rise in urinary sodium preceded the peak of glycosuria in each animal and in Rat 805, it preceded the appearance of any glucose in the urine. Thereafter, the excretion

values for sodium remained very constant until treatment with insulin was renewed whereupon there was a temporary decrease in the amount of urinary sodium for each rat.

Urinary potassium. In the rats which were injected with the cortical steroids there was an increase in urinary potassium. The peak of the rise was not sustained. When the injection of the steroid was stopped the level of urinary potassium fell to normal values. There was no clear tendency for insulin to affect the excretion of potassium in the animals having adrenal steroid diabetes. The withdrawal of insulin from the depancreatized animals was followed by a rise in urinary potassium. The peak of the rise was not sustained. When treatment with insulin was renewed there was a temporary decrease in the excretion of potassium.

Urinary chlorides. The injection of the cortical steroids caused an increase in the excretion of chlorides during the first twenty-four hours. The extent of change was less than for sodium. The withdrawal of insulin from the depancreatized animals was followed by a temporary rise in the excretion of chlorides, and the renewal of insulin injections by a temporary decrease below normal in the excretion of chlorides. With the exception of the initial response to the injection of the cortical steroids the changes in urinary chlorides paralleled changes in urinary potassium much more closely than changes in urinary sodium.

DISCUSSION

The results of this study confirm the earlier report of Ingle (1941) on the production of adrenal steroid diabetes in force-fed normal rats. It is known (Ingle, in press) that untreated normal rats can tolerate much larger amounts of the high carbohydrate diet than were administered in this study. The amounts of glucose which were excreted during the injection of the cortical steroids were much greater than can be accounted for on the basis of increased formation of carbohydrate from protein, which is in agreement with Ingle and Thorn (1941) and Ingle (1941). The possibility that the extra glucose was formed from fat deserves consideration but it remains to be shown that such large amounts of glucose can arise from fat if, indeed, any such intraconversion normally occurs at all. Rather than to postulate that all of the urinary glucose represents "overproduction," it is more simple to assume that some of the carbohydrate of the diet, all of which was normally utilized (oxidation, storage and conversion), now fails to be utilized.

The first purpose of this study was to test the hypothesis that the effect of the C-11-17-oxygenated cortical steroids in stimulating the catabolism of protein and its conversion to carbohydrate might be secondary to an inhibitory effect of the cortical steroids upon the utilization of carbohydrate just as the withdrawal of insulin from the

depancreatized animal results in failure of carbohydrate utilization and an increased gluconeogenesis from protein. In planning the experiments it was expected that insulin would overcome the inhibitory effect of the cortical steroids upon carbohydrate utilization and possibly restore the nitrogen balance to normal just as in pancreatic diabetes. Two unanticipated results tended to obscure the relationship between the utilization of carbohydrate and the extent of nitrogen loss in adrenal steroid diabetes. First, the animals having adrenal steroid diabetes were highly resistant to insulin and it can be argued that even those animals which were free of glycosuria during treatment with insulin were not completely normal in respect to the utilization of carbohydrate. Second, it was observed in these and other studies (Ingle and Kuizenga, unpublished data) that the initially elevated level of urinary nitrogen tended to decrease towards normal although the intake of the cortical steroids was constant.

The failure of insulin to affect clearly the elevated level of urinary nitrogen in animals having adrenal steroid diabetes can be regarded as non-crucial evidence that the increased loss of nitrogen which follows the injection of the cortical steroids is a primary effect of these hormones and not secondary to their "anti-insulin" action. It may also be significant that following the beginning of cortical hormone injections (Figures 1 to 5) the initial rise in excretion of nitrogen preceded the onset of glycosuria.

The strongest evidence that the cortical hormones have a primary effect in stimulating the catabolism of protein has come from the excellent studies of White and Dougherty (1944) and Dougherty and White (1944) on the dissolution of lymphoid tissue and lymphocytes under the influence of the adrenal cortical hormones. The onset of these changes is so rapid and the regression of lymphoid tissue so marked that it would seem difficult to relate them to either a failure in carbohydrate utilization or to a primary "anti-anabolic" action of the cortical hormones which was postulated by Albright (1943). However, it remains to be shown that the action of the cortical hormones upon the body proteins is limited to that of the lymphocytes and lymphoid tissue. The total amount of protein represented by these tissues is much less than is lost by the whole body during the prolonged administration of the cortical hormones. Either the action of the cortical hormones involves directly the proteins of other tissues or as suggested by White (personal communication) the continuous breakdown and resynthesis of all body proteins may result in a draining off of amino acids via the negatively balanced lymphoid tissue. It is possible that the action of the cortical steroids in stimulating protein catabolism is not limited to lymphoid tissue but that there is a gradient of responsiveness among the structural tissues of the body so that the lymphoid proteins and those of the liver are the most readily mobilizable.

It was noted in this and other studies (Ingle and Kuizenga, unpublished data) that the peak of nitrogen excretion occurred within four to six days following the beginning of cortical steroid injections. It is possible that the peak of nitrogen excretion and the subsequent decrease towards a normal balance is related to the breakdown and exhaustion of lymphoid proteins. Other adaptative mechanisms may be involved.

The question remains as to how the apparent effects of the cortical hormones upon gluconeogenesis and upon carbohydrate utilization are related. If it could be assumed that the severe glycosuria observed in these experiments represents an overproduction of carbohydrate from fat as well as protein it would be unnecessary to postulate that the cortical steroids interfered with the utilization of carbohydrate or with the action of insulin. If there is inhibition of utilization, is it due to a direct interference with the action of insulin or is the inhibitory action upon other mechanisms involved in the metabolism of carbohydrate? Can the "anti-insulin" action of the cortical steriods be related to the increase in anti-body titer which Dougherty, White and Chase (1944) have observed to occur following the injection of these hormones? White (personal communication) has suggested that under the influence of the cortical steroids a weakly antigenic protein such as insulin may become more strongly antigenic. On such a basis it may be possible to relate the protein catabolizing action of the C-11-oxygenated cortical steroids to the "anti-insulin," carbohydrate utilization inhibiting action of these compounds.

We do not have a satisfactory explanation for the fact that in two of the animals having adrenal steroid diabetes (Rats 809 and 502, Figures 2 and 5) the withdrawal of insulin was followed by a temporary disappearance of glucose from the urine. The experimental conditions were rigidly controlled and it is improbable that experimental errors were involved.

It was observed by Thorn, Engel and Lewis (1941) and by Ingle and Thorn (1941) that the C-11-17-oxygenated adrenal cortical steroids cause an increase in the excretion of sodium, chloride and potassium in non-adrenalectomized animals. In the present study it was shown that the increase in the excretion of sodium and chloride was temporary and that a normal balance was quickly restored. The loss of potassium was probably due, in part at least, to its release from the catabolized tissues. The loss of chloride may have been secondary to the loss of the cations. The mechanism whereby these cortical steroids cause a temporary increase in the excretion of sodium is not known. Is there a direct effect upon the kidney threshold or is the site of action elsewhere in the body? Ingle (1945) suggested that the loss of sodium might be secondary to the effect of the hormones upon the metabolism of protein and carbohydrate. Could this amount of sodium arise from the dissolution of lymphoid tissue

and lymphocytes? Most tissue cells contain but little sodium but we have found no data on the sodium content of lymphoid tissue. Inspection of the data (Figures 1 to 5) shows that the loss of sodium occurred within the first twenty-four hours and that during this interval no loss of glucose occurred and the rise in urinary nitrogen was small. Sodium balance had been restored by the time the excretion of glucose and nitrogen had reached a peak. This evidence does not support the hypothesis that the loss of sodium is directly dependent upon changes in the metabolism of protein and carbohydrate.

The depancreatized rats showed a similar temporary rise in the excretion of sodium and chloride when insulin was withdrawn. This rise and fall occurred prior to the peak of excretion of nitrogen and glucose. In Rat 805 (Figure 8), the rise in urinary sodium occurred prior to the onset of glycosuria or to a rise in urinary nitrogen. Such sodium loss would seem to differ in cause from that occurring when glycosuria is severe (Rat 502, Figure 5) or in diabetic acidosis. Can the temporary effect of the C-11-17-oxygenated adrenal cortical steroids upon sodium balance be due to their "anti-insulin" action? According to Ingle, Evans and Sheppard (1944) insulin shock also causes a sharp temporary increase in the excretion of sodium and chloride. This effect is illustrated by Rat 905 (Figure 9) which developed insulin shock on one day. Lewis, *et al.* (1942) noted a similar increase in the excretion of sodium in non-adrenalectomized dogs and rats which were subjected to anoxia. It is evident that increases in urinary sodium occur in a variety of conditions. It may be possible to extend the hypothesis of Lewis, *et al* (1942) and assume that the C-11-17-oxygenated steroids have a specific property of increasing the excretion of sodium and that an increased secretion of these compounds by the adrenal cortices in anoxia, insulin shock, insulin withdrawal, etc. is the cause of the loss of sodium in each of these experimental conditions. It is also possible that the effect of the cortical steroids is non-specific and that changes in the secretory activity of the adrenal cortices do not mediate the increased excretion of sodium which occurs during certain types of stress.

Two rats (704 and 809) which died during the administration of 17-hydroxycorticosterone showed gross pathologic changes. Deep ulcers were found in the pyloric portion of the stomach of each rat. In addition, the cecum of rat 809 had several ulcers, two of which had penetrated the wall. These observations indicate the need for a systematic appraisal of the damaging effects of the cortical steroids.

SUMMARY

Male rats of 300 to 325 grams weight were force-fed a high carbohydrate diet. Glycosuria was induced in normal rats by the administration of 5.0 mgm. per day of either 17-hydroxycorticosterone or 17-hydroxy-11-dehydrocorticosterone. The accompanying

changes in weight and urinary excretion products were compared to the changes which occurred in partially depancreatized rats following the withdrawal of insulin. The onset of glycosuria in either adrenal steroid or pancreatic diabetes was accompanied by a loss of weight, an increase in urinary nitrogen and potassium and a temporary increase in urinary sodium and chloride. When the administration of the steroid was stopped in the intact animals and when treatment with insulin was renewed in the depancreatized animals the changes from normal were reversed. Adrenal steroid diabetes but not pancreatic diabetes was highly resistant to control by insulin. The administration of insulin did not clearly affect the elevated level of urinary nitrogen in rats having adrenal steroid diabetes.

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INDUCTION OF ESTRUS IN THE OVARIECTOMIZED GOLDEN HAMSTER

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IT HAS BEEN REPORTED (McKenzie and Terrill, 1937; Bell, Casida, and Darlow, 1941; Quin and Van Der Wath, 1943) that the ovariectomized female sheep and goat are brought into estrus uniformly and promptly by a single injection of estrogen, a result confirmed at this Station (unpublished results). In this respect both animals differ from the common laboratory mammals (guinea pig, rat, and mouse) which require progesterone following estrogen priming for the regular exhibition of estrus. The marked difference in response to estrogen in the respective species indicates that the mechanisms involved in the induction of estrus are not identical. Since female sheep and goats exhibit a well-defined period of seasonal anestrum, it was thought that this factor might possibly be associated with the variations cited.

The descriptions of the breeding habits of the golden hamster given by Bruce and Hindle (1934), Deanesly (1938) and Peczenik (1942), and our own observation that it failed to breed from October to the latter part of January, suggested that this animal was characterized by a period of seasonal anestrum and might then respond as do the ovariectomized sheep and goat to estrogen. The present study was undertaken to test this possibility. Our results, however, indicate that the induction of estrus in the golden hamster is brought about essentially as in the rat, in the guinea pig, and in the mouse. While our findings do not therefore contribute to the solution of the original problem, it is believed that a report on this work will contribute to the general knowledge on the subject.

MATERIALS AND METHODS

Ninety-three female hamsters were spayed for the experiments. The ovaries with a portion of the adjacent fat, the fallopian tubes, and part of the uterine horns were removed under general anesthesia.[1] Forty of these females were 6 months old or older and the other 53 were just reaching sexual maturity when they were ovariectomized. None of 38 autopsied animals showed any sign of ovarian regeneration.

Each animal was used in a number of tests, the interval between tests

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[1] Our thanks are due to Dr. Irving Rothchild for ovariectomizing some of the animals.

ranging from 1 to several weeks. After the injection of estrogen, observations were made at various intervals during periods ranging from 17 to 72 hours.

Progesterone was injected either 24 or 48 hours, usually 48 hours, following the administration of estrogen. Observations following progesterone injection were generally made over a period of 9 to 17 hours. Since the males were found to be quite sluggish during the day, injections were timed where possible so that the females could be tested for estrus during the night hours.

As a control on each experiment with estrogen-progesterone a number of animals were treated with estrogen alone; these were tested at the same time as were the estrogen-progesterone treated animals.

The females were checked for estrus by placing each individual in a cage with one to two males.[2] They were left with the males until either a positive or a negative response could be determined. The criterion of estrus was directly observed copulation. Those females in estrus mated within a few minutes after being placed with the males. Those not in estrus either fought the males or avoided them while making a constant search for food.

The estrogen (crystalline estradiol benzoate) and the progesterone[3] were prepared for injection by dissolving in Mazola oil. All injections were given subcutaneously in the backs of the animals. A few animals developed purulent abscesses and were destroyed; a few others developed small oily cysts which were broken down by massage.

RESULTS

Estrogen Injections

Out of a total of 374 injections of estrogen alone, only 28 induced estrus (table 1). The estrus period in 23 of these was very short. The

TABLE 1. THE INDUCTION OF ESTRUS BY INJECTION OF ESTROGEN (CRYSTALLINE ESTRADIOL BENZOATE) IN THE OVARIECTOMIZED GOLDEN HAMSTER

Estrogen	Length of observation after estrogen injection	Injections	Injections followed by estrus	
gamma	*Hours*	*No.*	*No.*	*%*
0.04	24 to 37	31	0	0
0.2	24 to 56	44	2	4
1.0	24 to 72	67	4	6
5.0	24 to 72	75	2	3
10.0	24 to 72	13	4	30
20.0	17 to 65	57	4	7
25.0	24 to 72	80	11	13
100.0	24 to 72	7	1	14

animals allowed copulation only once during any observation period. Of the remaining 5 animals, 3 were in estrus at an initial trial and were negative for 13 to 29 hours thereafter, when they again exhibited estrus. The 2 remaining animals were in estrus for 6 and 12 hours respectively. Twenty-one of the 28 estrus responses occurred from

[2] We wish to thank Dr. O. N. Eaton for making available facilities for maintenance and testing many of the hamsters.

[3] Progynon B and Proluton, respectively, Schering Corporation, Bloomfield, New Jersey.

24 to 34 hours after injection. In 9 of these 21, mating occurred at the 24th hour, which was the earliest response observed.

Over a wide range of dosages, the amount of estrogen injected had little relation to the incidence of estrus. No animals came into estrus following the injection of 0.04γ and less than 10 per cent of all those receiving 0.2 to 5γ. Larger doses gave very erratic results. The greatest response, 30 per cent, was obtained with 10γ estrogen, 7 per cent with 20γ, and 14 per cent with 100γ.

Estrogen-Progesterone Injections

There was no appreciable difference in the estrus response of females to progesterone injected either 24 or 48 hours following estrogen. In most tests progesterone was administered 48 hours following estrogen.

When estrogen injections were followed with 0.02 mg. of progesterone estrus was not induced, while an increase of the progesterone to 0.05 mg. was as effective in inducing estrus as was 10 times this quantity (table 2). The small doses of estrogen, from 0.04 to 5γ,

TABLE 2. THE INDUCTION OF ESTRUS BY INJECTION OF ESTROGEN FOLLOWED BY PROGESTERONE IN THE OVARIECTOMIZED GOLDEN HAMSTER

Estrogen	Progesterone[1]	Injections	Injections followed by estrus	
gamma	*mg.*	*No.*	*No.*	*%*
0.04	0.5	35	13	37
0.2	0.02	16	0	0
0.2	0.1	13	4	30
0.2	0.2	9	1	11
0.2	0.5	40	19	47
1.0	0.1	25	15	60
1.0	0.5	56	20	35
5.0	0.5	47	22	47
10.0	0.05	11	11	100
20.0	0.02	13	0	0
20.0	0.05	23	23	100
20.0	0.1	14	14	100
20.0	0.5	60	51	85
25.0	0.5	50	49	98

[1] Progesterone injections were made either 24 or 48 hours following the administration of estrogen; since results were comparable for the two intervals they are combined in the table.

followed by progesterone at dosages of 0.1 to 0.5 mg. induced estrus in 11 to 60 per cent of the cases. With these doses all responsive animals were in estrus by the 6th hour. The estrus response terminated in some animals by the 10th to 12th hour, and in all by the 17th hour. Larger doses of estrogen, ranging from 10 to 25γ, when followed by 0.05 to 0.5 mg. of progesterone, induced estrus in 85 to 100 per cent of the animals. Following these doses all the animals remained in estrus beyond the 12th hour, but only 13 of 86 females were in estrus at the end of the 17th hour.

Twenty-five females that received either 10 or 25γ of estrogen followed by 0.1 to 0.5 mg. of progesterone were observed at hourly

intervals for the first 3 hours. Three were in estrus at the end of the first hour, an additional 18 at the end of the second hour, and 3 more at the end of the third hour. Under the conditions of these injections the latent period appears to be no more than 2 or 3 hours.

Control animals receiving estrogen and progesterone simultaneously did not exhibit estrus within at least 11 hours following injection. Likewise, animals receiving either Mazola oil or progesterone alone gave a negative response over a comparable period.

DISCUSSION

The data which have been presented indicate that the hormonal factors responsible for the occurrence of estrus in the hamster are essentially similar to those found for the guinea pig (Dempsey, Hertz and Young, 1936), rat (Boling and Blandau, 1939), and mouse (Ring, 1944). It may be of interest to compare the optimal dosages required by the hamster with dosages previously reported for other species. The hamster is apparently even less responsive to estrogen alone than are the other species. A dose of 10γ induced estrus in 30 per cent of the hamsters. In contrast, a dose of 1,000 I.U. (30γ) induced estrus in 65 per cent of treated guinea pigs, 100 R.U. (15γ) induced estrus in 90 per cent of the rats, and 100 R.U. (15γ) induced estrus in 80 per cent of the mice. The hamster, similar to the mouse, responded to a smaller dosage of progesterone than the guinea pig or rat. In both the mouse and hamster, it required 0.05 mg. of progesterone following estrogen priming to induce estrus as compared with the larger dose of 0.1 mg. for the guinea pig and 0.3 to 0.4 mg. for the rat.

Experimental results indicated that a 7 day rest period following estrogen-progesterone injections was not sufficient for the complete destruction or elimination of all residual hormone. Five γ of estrogen followed in 48 hours with 0.5 mg. of progesterone given after a 7 day rest period induced estrus in 88 per cent of the animals, but following a 14 day, or longer, rest period it induced estrus in only 47 per cent of the animals.

As was noted earlier, the tests reported here were initiated in the belief that the golden hamster exhibited a true seasonal anestrum. Subsequently, however, Kupperman (1944) stated that the hamster displayed regular cyclic activity throughout the year. More recently Bond (1945) has reported breeding activity throughout the year, although at a lesser intensity between June and October. It is difficult to reconcile these observations with the earlier accounts of Bruce and Hindle (1934), Deanesly (1938) and Peczenik (1942), and with the failure of breeding in our colony from October through January. Environmental factors most probably underlie the reported differences in reproductive behavior, but the precise variables apparently have not been defined. Genetic differences may, however, have arisen in the animals of different colonies with resultant differences in breeding behavior.

SUMMARY

Estrus was induced regularly in the ovariectomized golden hamster by the injection of 10γ of estrogen followed in 24 to 48 hours by the injection of 0.05 to 0.5 mg. of progesterone. The administration of estrogen alone over a wide range of dosages induced estrus in only a small percentage of animals. The estrous response following estrogen-progesterone was of longer duration and resembled estrus in the normal female.

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THE INFLUENCE OF GOITROGENIC COMPOUNDS (SULFONAMIDES AND THEIR DERIVATIVES, THIOUREA AND ITS DERIVATIVES) ON RESPIRATION OF THYROID TISSUE[1]

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AN *in vitro* reaction in which appreciable amounts of radioactive iodine are incorporated into diiodotyrosine and thyroxine by surviving thyroid slices in as brief a period as 2 hours (Morton and Chaikoff, 1943) has provided a useful tool for elucidation of the mechanism of action of goitrogenic compounds, substances that produce an enlargement and hyperplasia of the thyroid gland. This procedure revealed that such compounds as the sulfonamides, p-aminobenzoic acid, thiourea, thiouracil, etc., inhibit the incorporation of inorganic iodide into diiodotyrosine and thyroxine (Franklin and Chaikoff, 1944; Franklin, Chaikoff, and Lerner 1944).

In an earlier communication (Schachner, Franklin, and Chaikoff, 1943) it was shown that cyanide, azide, sulfide, and carbon monoxide inhibited the *in vitro* formation of diiodotyrosine and thyroxine from inorganic iodide by thyroid tissue. The inhibition produced by CO was more pronounced in the dark than in the light. Anaerobiosis caused a strong inhibition when the amount of O_2 was reduced below 0.6 per cent. On the basis of these findings it was concluded that the formation of both diiodotyrosine and thyroxine by the thyroid gland is linked with aerobic oxidations in which the cytochrome-cytochrome oxidase system is concerned. It therefore became of interest to determine whether goitrogenic compounds like p-aminobenzoic acid, the sulfonamides, thiourea and thiouracil exert their inhibitory effects upon the sythesis of diiodotyrosine and thyroxine by interfering with the cytochrome-cytochrome oxidase system.

EXPERIMENTAL

Preparation of slices—Thyroid glands were obtained from sheep weighing about 30 kg. at the local abattoir. They were sacrificed by exsanguination and their thyroid glands excised within 5 minutes thereafter. The glands were wrapped in waterproof cellophane and kept cool by surrounding the cellophane with cracked ice. In the laboratory, the glands were first rinsed

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with a Ringer-phosphate solution and sliced freehand with a razor blade. All slices obtained from a single lobe that weighed about 1.5 gm. were pooled in a vessel containing several cc. of a Ringer-phosphate solution. An interval of about 45 minutes elapsed between the time that the sheep were sacrificed and the time that the Warburg flask containing the thyroid slices was placed in the thermostat; during the first half of this interval the inact thyroid gland was kept chilled; during most of the remainder the slices were suspended in a Ringer-phosphate solution kept at room temperature.

Measurement of oxygen consumption—Thyroid slices were carefully selected with respect to uniformity of area and thickness. Fifty mg. of such slices were weighed in a micro-torsion balance and rapidly transferred to a Warburg flask containing 3.0 ml. of a Ringer-phosphate solution in the main part of the chamber and 0.2 ml. of the 20 percent KOH and a strip of filter paper in the central well. Oxygen consumption was measured in a pure O_2 atmosphere by the direct method of Warburg (Dixon, 1943) in a thermostat maintained at 37.8°. Manometer readings were made at 10-minute intervals after a 15-minute period had been allowed for temperature equilibration. Readings were continued for at least one hour. The results are expressed as QO_2 (cubic mm. of oxygen consumed per mg. of dry tissue per hour). To obtain the wet weight-dry weight ratio, 2 samples of slices from each gland weighing approximately 200 mg. were dried to constant weight at 90°.

Media—The phosphate-Ringer medium used here was prepared as follows: 100 parts of an isotonic solution of NaCl was mixed with 2 parts of each of isotonic solutions of KCl, $CaCl_2$, and $MgSO_4$. The mixture was boiled for 15 minutes and, while it cooled, was saturated with CO_2-free oxygen. The original volume was restored by the addition of boiled, distilled water saturated with oxygen. To 10 volumes of this solution was then added one volume of an isotonic phosphate buffer at pH 7.4, which had been boiled and saturated with oxygen as described above.

All compounds tested were dissolved in the mixture of NaCl, KCl, $CaCl_2$ and $MgSO_4$ just after it had been saturated with oxygen. Care was taken to adjust this solution to pH 7.4 with NaOH or HCl before the addition of the phosphate buffer.

RESULTS

In previous studies on the *in vitro* formation of thyroxine and diiodotyrosine, the thyroid slices were suspended in a bicarbonate-Ringer medium. Since the respiratory measurements recorded below were carried out in a phosphate-Ringer medium, it became necessary to determine whether compounds that inhibited synthesis in the presence of the bicarbonate medium were also effective when phosphate served as the buffer.

The data of the experiments labeled control (Table 1) show that the amounts of radiothyroxine and radiodiiodotyrosine formed when slices of sheep thyroid are suspended in a phosphate-Ringer medium are practically the same as those observed in the presence of the bicarbonate-Ringer. Moreover the results of Table 1 leave no doubt that thiourea, p-aminobenzoic acid, sulfathiazole and sulfanilamide are effective inhibitors of the synthesis of thyroxine and

diiodotyrosine by thyroid slices when suspended in a phosphate-Ringer solution.

The sulfonamides—The effect of sulfanilamide on respiration of thyroid slices was tested at 2 concentrations (Table 2). At a con-

TABLE 1. EFFECT OF GOITROGENIC SUBSTANCES ON THE FORMATION OF RADIOTHYROXINE AND RADIODIIODOTYROSINE BY SURVIVING SHEEP THYROID SLICES

(250 mg. of slices were incubated for 2 hours at 38° in a buffered Ringer solution containing inorganic iodide I^{131}. No. I^{127} was added.[1] The procedures employed here have been described elsewhere (Franklin, Chaikoff and Lerner, 1944).

Expt.	Compound added	Concentration	Buffer	Radioactive iodine of Ringer solution recovered as	
				Thyroxine	Diiodotyrosine
1	None	Control	phosphate	13.1	57.8
	None	Control	phosphate	12.7	60.1
	None	Control	phosphate	12.3	58.8
	None	Control	bicarbonate	11.1	62.8
	None	Control	bicarbonate	12.8	62.8
	None	Control	bicarbonate	11.9	54.5
	Thiourea	10^{-3}M	phosphate	3.9	9.2
	Thiourea	10^{-2}M	phosphate	3.9	8.1
	Thiourea	10^{-3}M	phosphate	4.1	9.9
	p-Aminobenzoic acid	10^{-3}M	phosphate	2.9	4.1
	p-Aminobenzoic acid	10^{-3}M	phosphate	2.9	4.1
	p-Aminobenzoic acid	10^{-3}M	phosphate	2.9	3.3
	Sulfanilamide	10^{-3}M	phosphate	8.9	46.8
	Sulfanilamide	10^{-3}M	phosphate	8.7	40.0
	Sulfanilamide	10^{-3}M	phosphate	8.1	44.4
2	None	Control	phosphate	6.8	30.6
	None	Control	phosphate	6.0	30.6
	None	Control	phosphate	6.3	29.6
	Sulfathiazole	10^{-3}M	phosphate	2.0	3.3
	Sulfathiazole	10^{-3}M	phosphate	1.8	3.6
	Sulfathiazole	10^{-3}M	phosphate	1.6	3.6
	Sulfanilamide	10^{-3}M	phosphate	3.6	19.2
	Sulfanilamide	10^{-3}M	phosphate	3.4	18.9
	Sulfanilamide	10^{-3}M	phosphate	3.7	22.8

[1] The only I^{127} contained in the reaction flask was that due to impurities in the reagent quality chemicals used in the preparation of the media.

centration of 10^{-3}M, which in an earlier report (Franklin and Chaikoff, 1944) as well as here was shown to depress diiodotyrosine and thyroxine formation, sulfanilamide had little or no effect on the uptake of oxygen by sheep thyroid slices. A tenfold increase in the concentration of sulfanilamide (10^{-2}M) did decrease the QO_2 by about 20 per cent. This depression in O_2 consumption, however, was small as compared with its effect on the synthesis of thyroxine and diiodotyrosine; in the presence of 10^{-2}M sulfanilamide, the conversion

of inorganic iodide to thyroxine and diiodotyrosine was almost completely inhibited.

Small decreases in QO_2 were observed in the presence of 10^{-3}M sulfapyridine and sulfathiazole. But here again, despite the fact that at this concentration the incorporation of inorganic iodide into diiodotyrosine and thyroxine is almost completely inhibited, the rate of O_2 uptake by the thyroid slices was not far from normal.

p-Amino aromatic acids—p-Aminobenzoic acid, p-aminophenylacetic acid, and sulfanilic acid were tested at a single concentration,

TABLE 2. THE EFFECT OF SULFONAMIDES ON THE QO_2 OF SURVIVING SHEEP THYROID SLICES IN PHOSPHATE-RINGER MEDIUM

Expt.	Sulfonamide added	Concentration	QO_2	
			Control	In presence of Sulfonamide
1	Sulfanilamide	10^{-2}M	4.9	3.8
2	Sulfanilamide	10^{-2}M	3.4	3.1
3	Sulfanilamide	10^{-2}M	3.6	2.8
4	Sulfanilamide	10^{-3}	3.5	3.3
5	Sulfanilamide	10^{-3}	3.6	3.3
6	Sulfanilamide	10^{-3}	4.3	4.2
7	Sulfanilamide	10^{-3}	4.0	3.9
8	Sulfapyridine	10^{-3}	3.2	3.0
9	Sulfapyridine	10^{-3}	2.4	2.6
10	Sulfapyridine	10^{-3}	3.0	2.7
11	Sulfathiazole	10^{-3}	4.2	4.0
12	Sulfathiazole	10^{-3}	3.2	2.9
13	Sulfathiazole	10^{-3}	2.4	2.0

namely 10^{-2}M (Table 3). The finding that these 3 compounds had no effect on respiration is indeed striking in view of the fact that even at 10^{-3}M these compounds were powerful inhibitors of the synthesis of diiodotyrosine and thyroxine by surviving sheep-thyroid tissue.

Aniline, which also strongly depresses the formation of diiodotyrosine and thyroxine, likewise failed to depress the rate of O_2 uptake.

Thiourea and related compounds—As noted elsewhere (Franklin, Chaikoff, and Lerner, 1944), thiourea, thiouracil and allylthiourea stopped the *in vitro* conversion of inorganic iodide to diiodotyrosine and thyroxine at concentrations as low as 10^{-3}M. The results recorded in Table 4 show that the mechanism of this inhibition is not dependent upon an interference in the uptake of O_2. Even at a concentration as high as 10^{-1}M, thiourea failed to depress the QO_2 of surviving thyroid tissue.

Cyanide and azide—(Table 5)—Cyanide and azide resemble the compounds listed in Tables 1–4 in that they also inhibited diiodotyrosine and thyroxine formation (Schachner, Franklin, and Chaikoff, 1943). They differ, however, from the other compounds in their

TABLE 3. THE EFFECT OF p-AMINO AROMATIC ACIDS ON THE QO_2 OF SHEEP THYROID SLICES IN PHOSPHATE-RINGER MEDIUM

Expt.	Compound added	Concentration	QO_2	
			Control	In presence of added compound
1	p-Aminobenzoic acid	10^{-2}M	3.2	3.1
2	p-Aminobenzoic acid	10^{-2}M	3.8	3.7
3	p-Aminobenzoic acid	10^{-2}M	3.5	3.1
4	p-Aminophenylacetic acid	10^{-2}	4.3	4.4
5	p-Aminophenylacetic acid	10^{-2}	2.3	2.4
6	Sulfanilic acid	10^{-2}	4.3	4.2
7	Sulfanilic acid	10^{-2}	3.8	3.8
8	Aniline	10^{-3}	2.3	2.3
9	Aniline	10^{-3}	4.4	4.4

TABLE 4. THE EFFECT OF THIOUREA AND RELATED COMPOUNDS ON THE QO_2 OF SURVIVING SHEEP THYROID SLICES IN PHOSPHATE-RINGER MEDIUM

Expt.	Compound added	Concentration	QO_2	
			Control	In presence of added compound
1	Thiourea	10^{-1}M	2.5	2.2
2	Thiourea	10^{-2}	2.5	2.5
3	Thiourea	10^{-3}	3.3	3.3
4	Thiourea	10^{-2}	6.4	6.1
5	Thiourea	10^{-3}	3.3	3.5
6	Thiouracil	10^{-2}	2.7	2.4
7	Thiouracil	10^{-3}	2.7	3.0
8	Thiouracil	10^{-3}	3.9	3.7
9	Allylthiourea	10^{-3}	2.5	2.4
10	Allylthiourea	10^{-2}	3.5	3.3
11	Allylthiourea	10^{-2}	3.0	2.5

TABLE 5. THE EFFECT OF AZIDE AND CYANIDE ON THE CO_2 OF SHEEP THYROID SLICES IN PHOSPHATE-RINGER MEDIUM

Expt.	Compound added	Concentration	QO_2	
			Control	In presence of added compound
1	Cyanide*	10^{-3}M	2.5	0.3
2	Cyanide	10^{-3}M	3.2	0.6
3	Azide	5×10^{-3}	3.0	1.9
4	Azide	5×10^{-3}	4.5	3.0

* In order to prevent the absorption of HCN from the medium by the alkali insets, an absorbent modified according to Krebs (1935) was used. It consisted of a mixture of 10 parts in KOH and 1 part in KCN.

effects on respiration *in vitro* by thyroid tissue. In the presence of 10^{-3}M cyanide, the uptake of O_2 by thyroid slices almost ceases. Azide at 5×10^{-3}M depressed the QO_2 by about 40 per cent.

DISCUSSION

The observation that both cyanide and azide greatly depress the oxygen consumption of surviving thyroid slices is in line with earlier evidence obtained in this laboratory in support of the view that the cytochrome-cytochrome oxidase system plays a part in the over-all reaction by which inorganic iodide is incorporated into diiodotyrosine and thyroxine by thyroid tissue (Schachner, Franklin, and Chaikoff, 1943). But an interference in this enzyme system could not account for the inhibitory action of such compounds as the sulfonamides, p-aminobenzoic acid, thiourea, thiouracil, etc., upon the synthesis of diiodotyrosine and thyroxine. In the presence of a concentration (10^{-3}M) that depressed the formation of diiodotyrosine and thyroxine to the extent of 60–85 per cent (Franklin and Chaikoff, 1944; Franklin, Chaikoff, and Lerner, 1944) these compounds had little or no effect on the oxygen uptake of thyroid slices. Thioruea failed to depress oxygen consumption even at a concentration as high as 10^{-1}M. In a single experiment, 10^{-2}M sulfanilamide depressed respiration of thyroid slices by about 30 per cent. In general, however, the reduction in respiration was in no way comparable with the extent to which the synthesis of diiodotyrosine and thyroxine was inhibited.

Jandorf and Williams (1944) reported that the respiration of thyroid glands excised from rats that had been fed thiouracil was increased. This finding, however, is not surprising, since the goitrogenic action of that compound results in an increase in the cell volume of the thyroid follicle. The adrenal glands of their thiouracil-treated animals showed no increase in respiration. Řeřábek (1943) measured the respiration of the following tissues that had been excised from mice fed a single dose of sulfanilamide: liver, kidney, brain, heart, skeletal muscle, diaphragm, intestinal epithelium, and bone marrow. Little or no effect on the oxygen uptake of these tissues was produced by the administered sulfanilamide.

In view of the association between inhibitory activity and ease of oxidizability among the sulfonamides and their derivatives (Taurog, Chaikoff, and Franklin, 1945) the indications are therefore strong that these compounds interfere with an oxidative reaction concerned with the synthesis of diiodotyrosine and thyroxine. Support for this concept is provided by the finding that without a single exception all active compounds so far examined inhibited an oxidative reaction, namely the conversion of inorganic iodide into diiodotyrosine-iodine, a reaction that at the present time is regarded as a preliminary step

in the formation of thyroxine. Although the results of the present investigation fail to identify the oxidative system affected by the inhibitors, they do serve to rule out the cytochrome-cytochrome oxidase system.

SUMMARY

In view of the fact that it was shown earlier that the cytochrome-cytochrome oxidase system is concerned in the sythesis of diiodotyrosine and thyroxine, a study was made to determine whether an interference with this enzyme system could account for the inhibitory effects of the sulfonamides and their derivatives and of thiourea and its derivatives upon the incorporation of inorganic iodide into diiodotyrosine and thyroxine by surviving thyroid slices.

No marked effect upon the oxygen consumption of surviving sheep-thyroid slices was observed when they were suspended in a Ringer medium containing the sulfonamides, or the p-amino aromatic acids, or thiourea, or thiouracil, or allylthiourea. It is therefore concluded that these compounds do not interfere with the cytochrome-cytochrome oxidase system in thyroid tissue.

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ACTIVITY OF SYNTHETIC ESTROGENS ON ORAL ADMINISTRATION IN THE DOMESTIC FOWL AND TURKEY

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JAAP AND THAYER (1944) reported that the dimethyl ether of diethylstilbestrol was a much more active estrogen for oral administration in the domestic fowl than diethylstilbestrol, its diethyl ether or its dipropionate. This result was in distinct contrast to that observed in rats and mice (Sondern, Sealey and Kartsonis, 1941), where the oral activity appeared to decrease with increase in the length of the chain in the alkoxy groups. Due to the practical significance of estrogens in the fattening of poultry (Jaap and Thompson, 1945; Thayer, Jaap, and Penquite, 1945), it appeared desirable to explore the oral activity of a number of synthetic compounds known to have some estrogenic activity in mammals.

The relative quantity of an estrogenic substance required to produce estrus in the rat and mouse varies with the compound. Using the same injection technique, Laqueur (1935) found that rats required ten to sixteen times as much estrone as mice to produce a certain stage of estrus in two-thirds of the injected animals. In contrast, the mouse required four times as much estradiol monobenzoate as the rat to produce an equivalent estrogenic response. Sondern and Sealey (1940) observed remarkable species differences in the response of rats and mice to estrone, estradiol, estriol and diethylstilbestrol both orally and injected. Since the relative response to different estrogens is not similar in rats and mice, and since the economic importance of estrogenic fattening should be greatest in the domestic fowl and turkey, these two species of birds were chosen for the oral assays.

MATERIALS AND METHODS[1]

The estrogenic compounds tested by oral assay in the domestic fowl and turkey may be structurally illustrated as follows:

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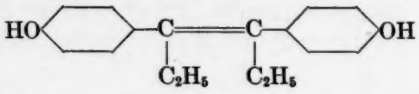
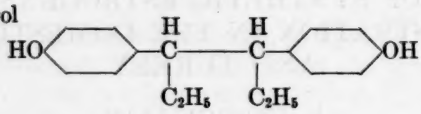
[1] Several companies generously supplied the chemicals used in these tests. The companies and the compounds supplied were as follows: George A. Breon & Co. I, IV, VII, and VIII; White Laboratories, Inc. I, II, III, IV, V, and VI; Mallinckrodt Chemical Works IX, X, XI, XVI, and XVII; Schieffelin & Co. XIV and XV; and Wallace & Tiernan Products Inc. XII and XIII. A part of the financial support for this investigation was received from White Laboratories Incorporated.

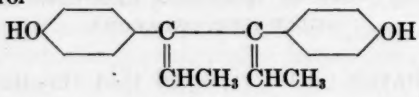
I. Diethylstilbestrol



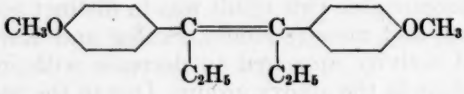
II. Hexestrol



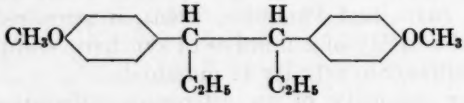
III. Dienestrol



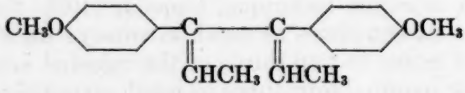
IV. Dianisylhexene



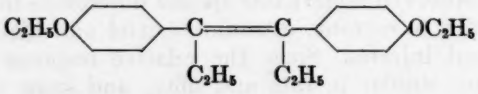
V. Dianisylhexane



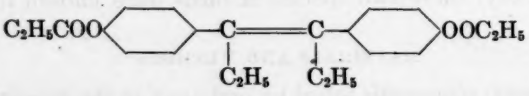
VI. Dianisylhexadiene



VII. Diethyl ether of diethylstilbestrol

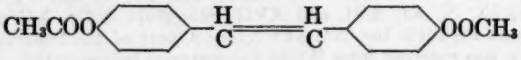


VIII. Dipropionate of diethylstilbestrol



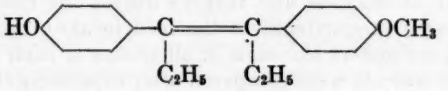
IX. Dihydroxystilbene

X. Diacetate of dihydroxystilbene

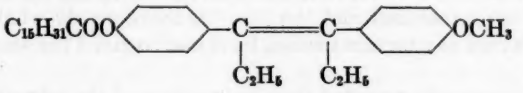


XI. Dimethyl ether of dihydroxystilbene

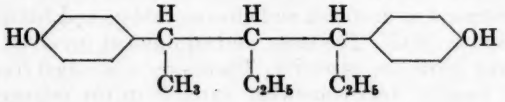
XII. Monomethyl ether of diethylstilbestrol



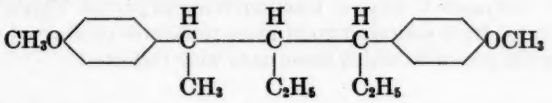
XIII. Monopalmitate of XII



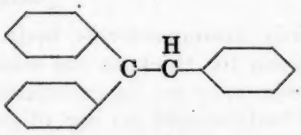
XIV. Benzestrol



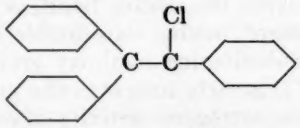
XV. Dimethyl ether of benzestrol



XVI. Triphenylethylene



XVII. Triphenylchloroethylene



The test for estrogenic activity used in these studies was the increase in oviduct weight by the seventeenth day after hatching. Female chicks and turkey poults were fed a mash mixture containing the estrogen from hatching time for 16 days and killed on the seventeenth day. The estrogens were dissolved in a sufficient quantity of soybean oil to make 5 cc. of oil per pound of feed. This oil solution was then thoroughly mixed with the mash feed which was fed *ad libitum*.

A part of the chicks received a higher protein feed than that fed to the others. This feed, containing more crude protein, was the same as that given to the turkey poults. Those chicks receiving the turkey feed grew more rapidly and were larger at the seventeenth day when they were killed to secure oviduct weights.

Compounds IX, X, XI, XVI, and XVII were tested with White Leghorn chicks. Oklabar chicks, an auto-sexing variety maintained at the Oklahoma Agricultural Experiment Station, were used for all other tests on the domestic fowl. The turkey poults were of four different varieties; Bronze, White, Slate, and Naragansett.

Growth rate of chickens and turkeys during the first few weeks after hatching is closely proportional to the feed intake. Average initial body weight was approximately the same in all groups of each species. For these reasons oviduct weights were compared after expressing them as mg. of oviduct per 100 gm. of body weight at the seventeenth day. By comparing the records in this manner, differences in estrogen intake during the sixteen-day feeding period are minimized and the result is more nearly what might be expected if each bird at a certain feeding level had received the same amount of estrogen.

It has been previously reported that assimilation of the estrogen appears to be much greater when it is evenly dispersed as an oil solution in the feed than when incorporated in starch and glucose tablets and fed daily (Thayer, Jaap and Penquite, 1945). The labor and equipment involved in controlled feeding of young birds are excessive. Therefore, controlled feeding was not used. Oviduct weights were somewhat variable in the estrogen stimulated groups.

An effort was made to have at least ten females per lot. Those lots having 20 or more individuals contain two or more replicates conducted at different times during the period in which these data were collected.

RESULTS

Chickens

The number of birds, hormone levels, body weights and relative potency of each estrogen for chickens are summarized in Table 1. The figures for relative potency are the calculated increases in oviduct weight per 100 gm. of body weight per mg. of estrogen in each pound of feed. Starting with diethylstilbestrol (I), it will be observed that hexestrol (II), which lacks the double bond, is slightly more active. Compound III, dienestrol, having two double bonds is more active than either I or II. Substituting methoxy groups for the hydroxyl radicals (IV, V and VI) greatly improves the potency of compounds I and II but reduces the estrogenic activity of compound III.

At the 10 mg. level dianisylhexane (V) is more active than dianisylhexene (IV); however, when the dosage level is increased to 30 mg. per pound of feed their relative activity is reversed. Unpublished data on fattening potency at the higher level with older chickens indicate that dianisylhexene (IV) is the more active of these two compounds. Reversal of relative potencies at different dosage levels was not observed in any of the other compounds. In general, most of the compounds produced a relatively greater increase in oviduct weight at the higher dosage levels.

The diethyl ether of diethylstilbestrol (VII) is probably less active than diethylstilbestrol. Diethylstilbestrol dipropionate (VIII) is approximately equivalent to dienestrol (III). Increasing the length

of chain on the para positions of the phenyl groups, therefore, appears to produce variable results. A test of the monomethyl ether of diethylstilbestrol (XII) demonstrates that it is only slightly more active than diethylstilbestrol. The high activity of compound IV is dependent on both methoxy groups being present. Compound XIII, in which a palmitate radical is substituted for the hydroxyl group in compound XII, shows no estrogenic activity at the level fed. A part of the inactivity of compound XIII may be due to the fact that it was secured in the form of starch-glucose tablets which were ground in a mortar and then mixed in the feed. Some estrogenic activity might have been observed if this compound had been dissolved in oil. Compound XIII is probably much less active than diethylstilbestrol because 0.5 mg. tablets of the latter over a ten-day period produced a 50 per cent increase in oviduct weight (Jaap and Thayer, 1944).

To further explore the oral estrogenic activity of stilbene derivatives, dihydroxystilbene (IX), its diacetate (X) and its dimethyl ether (XI) were tested at the 10 and 30 mg. levels. Dihydroxystilbene and its acetate are only slightly soluble in soybean oil and were, therefore, mixed in the feed partly as oil suspension. The dimethyl ether (XI) was somewhat more soluble but was not completely dissolved by the soybean oil. It will be observed from the data in Table 1 that these three compounds were completely inactive at the levels used in these trials.

Two additional estrogenic compounds containing two benzene rings were tested. Benzestrol (XIV) at 50 and 90 mg. levels per pound of feed showed only slight estrogenic activity. The dimethyl ether of benzestrol (XV) fed at a lower level (20 mg.) failed to produce any increase in oviduct weight.

Triphenylethylene (XVI) failed to produce any measurable estrogenic effect at the levels fed but triphenylcholoroethylene (XVII) exhibited distinct estrogenic activity approximately intermediate between that of hexestrol (II) and dienestrol (III).

Turkeys

Since the physiology of the turkey is very similar to that of the chicken, it would be expected that the oral activity of estrogenic compounds might be similar in these two species. The data presented in Table 2 show general similarity to those for the chicken (Table 1) but distinct differences in the relative potency of a few compounds exist. The dienestrol (III) was found to be slightly less active than either diethylstilbestrol (I) or hexestrol (II). These two latter compounds were relatively equal in the turkey whereas hexestrol showed greater potency in the chicken.

Whereas dianisylhexane at the lower level was most active of the compounds tested in the chicken, it was relatively inactive when orally administered in the turkey. In the turkey, dianisylhexene (IV) was a much more active estrogen than any of the others tested. The

monomethyl ether of diethylstilbestrol (XII), however, was much less active than diethylstilbestrol. The relative potency of most of the other compounds was similar to that for chickens. Triphenylchloroethylene (XVII) was found to be almost as active as diethylstilbestrol and hexestrol in the turkey. In the chicken triphenylchloroethylene was the fourth most active estrogen.

A test of the value of dissolving estrogens in oil was made with

TABLE 1. SUMMARY OF ORAL ASSAYS OF ESTROGENIC ACTIVITY IN CHICKENS

Compound designation	Reference name	Mg. of estrogen per lb. of feed	No. of chicks	Average body weight	Relative[1] potency
				(gm.)	
	No estrogen	0	122	131	0
I.	Diethylstilbestrol	23	21	117	2.1
		30	19	174	1.5
		50	30	87	2.9
II.	Hexestrol	30	16	126	3.4
III.	Dienestrol	10	11	153	5.0
		30	11	147	11.5
IV.	Dianisylhexene	10	46	107	20.9
		20	44	115	45.0
		50	8	112	41.9
V.	Dianisylhexane	10	48	118	32.0
		20	28	155	32.3
		30	8	170	37.8
VI.	Dianisylhexadiene	10	10	152	1.0
		30	10	158	4.4
VII.	Diethyl ether of diethylstilbestrol	10	17	132	0.5
		20	14	122	1.2
VIII.	Dipropionate of diethylstilbestrol	50	8	126	6.5
IX.	Dihydroxystilbene	30	16	142	0
X.	Diacetate of dihydroxystilbene	30	18	143	0
XI.	Dimethyl ether of dihydroxystilbene	30	15	136	0
XII.	Monomethyl ether of diethylstilbestrol	30	15	128	3.4
XIII.	Monopalmitate of XII	10	17	157	0
XIV.	Benzestrol	50	18	100	0.2
		90	10	110	0.2
XV.	Dimethyl ether of benzestrol	20	17	174	0
XVI.	Triphenylethylene	30	17	136	0
XVII.	Triphenylchloroethylene	30	17	138	8.5

[1] Increase in oviduct weight per 100 gm. of body weight per mg. of estrogen in each pound of feed.

turkeys. Three compounds (XI, XVI, and XVII) were mixed in the feed without the soybean oil and at a higher concentration, 50 mg. per pound (Table 2). Of these, the greater activity of the oil solution is readily evident for triphenylchloroethylene. When the crystals were mixed in the feed at the 50 mg. level, oviduct weight was 31 mg. per 100 gm. of body. As an oil solution dispersed in the feed 30 mg. of triphenylchloroethylene per pound of feed caused oviduct hypertrophy to 294 mg. per 100 gm. of body weight. Like diethylstilbestrol and its dimethyl ether, triphenylchloroethylene dissolved in oil has a much greater estrogenic potency for oral administration than when it is mixed in the feed in the crystalline state.

DISCUSSION

These studies of the oral activity of estrogens were conducted with the hope that some consistent relation might be observed between chemical composition or structure and oral estrogenic activity of the compound. Whereas converting the two hydroxyl groups into their methyl ethers enhanced the activity of one compound, it reduced activity of other very similar compounds. All compounds but XIV and XV may be considered as derivatives of stilbene. Within these fifteen

TABLE 2. SUMMARY OF ORAL ASSAYS OF ESTROGENIC ACTIVITY IN TURKEYS

Compound designation	Reference name	Mg. of estrogen per lb. of feed	No. of poults	Average body weight	Relative[1] potency
				(gm.)	
	No estrogen	0	42	136	0
I.	Diethylstilbestrol	30	9	156	10.5
		50	14	192	20.5
II.	Hexestrol	30	14	149	11.2
III.	Dienestrol	10	8	171	4.5
		30	14	196	8.3
IV.	Dianisylhexene	10	25	167	70.0
		20	15	175	70.0
V.	Dianisylhexane	10	13	147	0.6
		20	14	148	0.4
		30	15	182	0
VI.	Dianisylhexadiene	10	14	189	0
		30	7	186	0
VII.	Diethyl ether of diethylstilbestrol	10	15	144	3.3
VIII.	Dipropionate of diethylstilbestrol	10	12	130	4.8
IX.	Dihydroxystilbene	30	8	150	0
X.	Diacetate of dihydroxystilbene	30	8	175	0
XI.	Dimethyl ether of dihydroxystilbene	30	11	185	0
		50[2]	11	184	0
XII.	Monomethyl ether of diethylstilbestrol	10	11	119	1.4
XIII.	Monopalmitate of XII	10	12	192	0
XIV.	Benzestrol	50	18	96	0.1
		89	10	110	0.2
XV.	Dimethyl ether of benzestrol	20	12	166	0
XVI.	Triphenylethylene	30	11	153	0
		50[2]	13	190	0
XVII.	Triphenylchloroethylene	30	12	181	9.0
		50[2]	15	178	0.2

[1] Increase in oviduct weight per 100 gm. of body weight per mg. of estrogen in each pound of feed.

[2] Mixed in feed in crystalline form, not dissolved in soybean oil.

derivatives of stilbene, predictions of possible estrogenic activity by the oral route have not been possible. Whether further study of the derivatives of stilbene will show a relation between structure and physiological activity remains for further exploration.

Much data is needed concerning the fate of these chemicals in the digestive tract and within the body of the bird. Whether it be the higher metabolic rate of the bird or changes in storage organs such as the crop, the inactivation of estrogens or the activation of proestrogens in the bird differs greatly from that in the mouse and the rat.

The fact that solutions in a vegetable oil are more active indicates that either the oil acts as a protection against inactivation of the estrogen or the estrogen may be absorbed from the intestine along with lipids and thereby exerts an effect in the lymph system before being inactivated in the liver.

More exploration is needed on the derivatives of triphenylethylene (α-phenylstilbene). The mere substitution of a chlorine for a hydrogen atom in this compound has produced a relatively active estrogen for oral administration in birds. Further work is planned with compounds having substitutions and additions to this triphenylethylene nucleus. Such compounds as that designated as D. B. E. (Robson and Schönberg, 1942; Robson and Ansari, 1943) have been found to produce a more prolonged action when given orally to mice. The work of Robson and co-workers on this compound suggests that activity of an estrogen may be largely the result of its resistance to inactivation within the body. This may be the major factor in birds although limited comparisons of estrogens by the subcutaneous route (unpublished) indicate that some inactivation as well as absorption in the digestive tract may be involved.

SUMMARY

Seventeen synthetic estrogens have been tested for oral activity in the domestic fowl and turkey. All but two of these may be considered as derivatives of stilbene. Dianisylhexane was most active at the level of 10 mg. per pound of feed in the chick but practically inactive in the turkey poult. At levels of 20 or more mg. per pound of feed the dimethyl ether of diethylstilbestrol was the most potent estrogen for oral administration in both species. Compounds estrogenically inactive at the levels fed were dihydroxystilbene, its diacetate and its dimethyl ether; the dimethyl ether of benzestrol; and triphenylethylene. In contrast triphenylchloroethylene was relatively active on oral administration in both species. Further data were obtained showing that estrogenic activity of these chemicals is greatly increased by solution in soybean oil before dispersion in the feed.

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NOTES AND COMMENTS

THE EFFECT OF INSULIN ON THE SURVIVAL TIMES OF EVISCERATED RATS

This laboratory has undertaken studies of the effects of certain hormones upon the ability of eviscerated and noneviscerated rats to remove glucose from the blood. This study has to do with the range of insulin dosage which causes changes in the time of survival and in the rate of fall of blood glucose in the eviscerated rat.

METHODS

Male rats of the Sprague-Dawley strain were used. The diet was Purina Dog Chow. When the rats reached a weight of 180 to 190 grams the inferior vena cava was ligated between the liver and kidneys in order to cause the development of a collateral circulation. Asepsis was preserved during this operation. When the animals reached a weight of 250 grams they were anesthetized (intraperitoneal injection of 18 mgm. of cyclopentenyl-allyl-barbituric acid sodium) and eviscerated by the procedure described by Ingle and Griffith (1942). The animals were not fasted. All of the intra-abdominal organs were removed except the adrenals and the kidneys.

Intravenous infusions of a 0.9 per cent sodium chloride solution with and without insulin were made by a continuous injection apparatus which delivered fluid from each of two syringes at the rate of 20 cc. in twenty-four hours. The apparatus was powered by a synchronous motor and the reduction of motion was achieved by a precision built system of gears and levers so that an exact control of the rate of injection was attained.

The infusions were made into the saphenous vein of the right hind leg and were started within twenty minutes following the removal of the liver. The animals were secured in a supine position on an animal board. In these liverless animals one initial dose of the barbiturate was sufficient to maintain effective anesthesia throughout the experiment.

The analyses of blood glucose were made by the method of Miller and Van Slyke (1936). Samples of blood were taken from the tail just prior to the beginning of infusions and two, six and twenty-four hours later in those animals which survived. Samples of blood were taken from the jugular vein at the time of death.

EXPERIMENTS AND RESULTS

The data showing the relationship between insulin dosage, the time of survival and the level of blood glucose at time of death are summarized in Figure 1. Ten rats received infusions of saline only and ten rats were tested at each level of insulin dosage. The smallest amount of insulin injected represented one-sixty-fourth of a unit per twenty-four hours. Fourfold increases

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in insulin dosage were made in each step so that the largest amount represented 256 units of insulin per twenty-four hours.

The eviscerated animals which did not receive insulin had an average survival time of $1263 \pm$ seventy-six minutes. The administration of insulin at the rate of one-sixty-fourth of a unit per twenty-four hours was associated with shorter survival times which average 1050 ± 106 minutes. The ratio between the difference (213) and the standard deviation of the difference

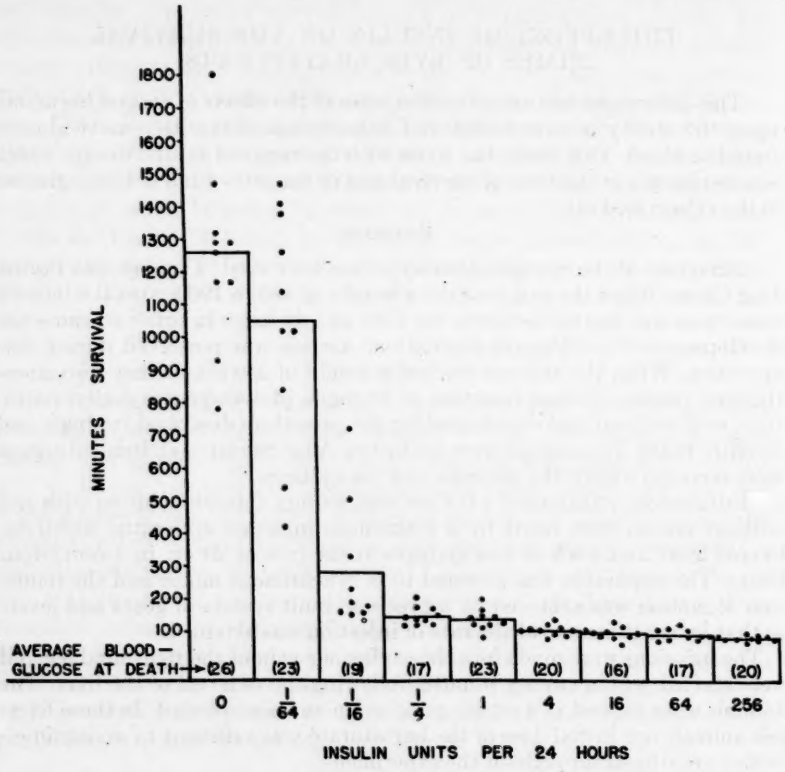

FIG. 1

(130.4) is 1.63 which, from a statistical standpoint, indicates the chances to be 95 in 100 that the true difference is greater than zero. It is, therefore, probable but not certain that this dose of insulin affected survival. Further increases in insulin dosage brought significant decreases in the average time of survival until a dosage level of 4 units per twenty-four hours was reached whereupon the response curve leveled off.

In respect to changes in blood glucose, the animals which did not receive insulin showed an average decrease of 42.7 ± 1.72 mgm. per cent within the first two-hour period. The administration of insulin at the rate of one-sixty-fourth of a unit per twenty-four hours was associated with a sharper average decrease, 49.9 ± 3.17 mgm. per cent within the first two-hour period. The

ratio between the difference (7.2) and the standard deviation of the difference (3.6) is 2.0 which, from a statistical standpoint, indicates the chances to be 98 in 100 that the true difference is greater than zero. It is therefore probable but not certain that this dose of insulin affected the level of blood glucose. When insulin was administered at the rate of one-sixteenth of a unit per twenty-four hours the average decrease in blood glucose at the end of two hours was 54.4 mgm. per cent and with a dose of one-fourth of a unit of insulin per twenty-four hours the average fall in blood glucose was 59.2 mgm. per cent at the end of two hours. There was no clearly established tendency for the blood glucose value at death to be related to the time of survival. The slightly higher average values in those animals which died after several hours may have been owing to chance or to the accumulation of non-fermentable reducing substances in the blood.

DISCUSSION

Mann and Magath (1923) were the first to demonstrate the hypoglycemic action of insulin in the hepatectomized dog. A number of investigators have published confirmatory data on the dog and other animals. These studies on the eviscerated rat show the marked effect of insulin upon survival time and that small amounts of insulin can accelerate the rate of fall of blood glucose. If it is assumed that a true difference was caused in the rate of fall of blood glucose during a two-hour period by the injection of insulin at the rate of one-sixty-fourth of a unit per twenty-four hours it can be stated that one in 768 parts of a unit affected the rate at which glucose was removed from the blood. The difference between the minimum effective dose and the dose which had an optimal effect upon blood glucose and survival was large.

This study confirms the observation of Ingle, Sheppard and Winter (1945) that the continuous intravenous infusion of the eviscerated rat with a solution of sodium chloride permits a prolonged survival. The amounts of glucose in the blood of these animals at time of death were very small. We have recorded values as low as 9 mgm. per cent and the average values have usually been less than 20 mgm. per cent. These values were probably higher than the values for true glucose due to the presence of non-fermentable reducing substances in the blood.

SUMMARY

Male rats were eviscerated at a weight of 250 grams and were given intravenous infusions of 0.9 per cent sodium chloride solution with and without added insulin until the time of death. There was an inverse relationship between insulin dosage and time of survival. Animals which did not receive insulin had an average survival time of 1263 minutes whereas optimal amounts of insulin shortened the time of survival to an average of 81 minutes. The effect of insulin in accelerating the rate at which glucose was removed from the blood paralleled the changes in the time of survival.

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TESTOSTERONE PROPIONATE INDUCES SECRETION OF MAMMARY GLANDS OF SUCKLING FEMALE RATS

Injection of suckling female rats with testosterone propionate causes enlargement of the nipples and growth and secretion of the mammary glands. This effect is established during the third week of life. The development of the mamma is in proportion to the size of the nipple, but there is wide variation in the speed of reaction not only between different animals but even between the glands of the same rat. Functioning glands continue to grow, so that prolonged treatment leads to a very pronounced response.

This response of the nipple of infantile rats to androgen was seen in a large number of baby albino rats during experiments with male hormone which were carried out in the Department of Obstetrics and Gynecology of the University of Michigan. The reaction of the mammary gland was therefore studied in 14 litters of albino rats comprising 21 experimental females. When many young rats were available, the controls were raised separately from their littermates which were used as experimental animals. In such cases, at the start of the experiment, the litters were recombined into a control and experimental group of 8 rats with siblings in both. Each group was placed with a mother rat and (except rats T 2 and T 3, see Table 1) left with it throughout the experiment. In 5 other rats treatment was started after weaning.

Experimental animals were injected subcutaneously with ½ mg. testosterone propionate (Oreton Schering) 3 times a week. In 16 suckling females treatment was begun at 2–8 days of age and continued to the 14th–30th day of life (Table 1a). Among the weaned rats in 2 animals treatment was started 3 days after weaning and lasted from the 21st to the 35th day of life; in one rat treatment was started 4 days after weaning at 21 days and lasted to the 35th day. The two oldest animals were injected for 2 weeks when they had reached a weight of 76 and 78 g; early in the experimental period the vagina opened in controls and test animals. Biopsies and necropsies were taken of the mammary glands, fixed in formalin or Bouin's mixture, defatted in xylene stained in toto with hemalum, and mounted in balsam. Similar preparations were made of 18 untreated females. The mammae of 6 hooded Long Evans strain rats[1] which had been injected with ½ mg. testosterone at more frequent intervals than in the Michigan experiments were also examined on whole amounts and on sections. Three young females had received 10 injections between the 7th and 22nd day, 2 others had received 10 injections between the 5th and 22nd day, 1 was weaned at 23 days and received 11 injections from the 24th to the 37th day.

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[1] I am greatly indebted to Dr. Earl T. Engle for repeating my experiments and for the data presented in Table 1b.

In suckling controls, the mamma consists only of a few ducts with club-shaped ends. There are no acini or acinar buds (Turner 1931). When androgen is injected, small acini are soon seen crowding around the ends of the ducts. Secretory acini were first seen in a two week old rat; on the whole mount preparation they appeared as clusters of delicate round vesicles. At this age, the mammary alveoli of 2 other treated rats were still in the proliferative stage and almost solid. Such individual variations continue through the third week; they are not related to differences in treatment such as the

TABLE 1A. SUCKLING FEMALE RATS, INJECTED 3 TIMES A WEEK WITH ½ MG. TESTOSTERONE PROPIONATE

Rat	Treated		Total dose mg.	Mammary gland
	from Day	to Day		
T 40	2	14	2½	immature
T 27	7	14	1½	proliferating
T 28	7	14	1½	secreting
T 41	2	17	3½	secreting
T 33	5	19	3	secreting
T 17	5	20	3	proliferating
T 20	5	22	3½	secreting
T 29	7	23	3½	proliferating
T 30	7	23	3½	secreting
T 32	5	25	4½	secreting
T 14	7	29	3	secreting
T 22	5	29	5½	secreting
T 23	5	29	5½	secreting
T 19	5	30	5½	proliferating
T 2[1]	8	30	3½	secreting
T 3[1]	8	30	3½	proliferating

Total 16 rats, secreting mammae in 10 of these.
[1] Injected 3 times a week with ½ mg. Neohombreol Roche, weaned at 23 days.

TABLE 1B. SUCKLING FEMALE RATS GIVEN 10 INJECTIONS OF ½ MG. TESTOSTERONE

Rat	Treated		Total dose mg.	Mammary gland
	from Day	to Day		
N. Y. 2	7	22	5	secreting
N. Y. 3	7	22	5	secreting
N. Y. 4	7	22	5	secreting
N. Y. 5	5	22	5	secreting
N. Y. 6	5	22	5	secreting

dose or the age at which the first injection was given. By the fourth week, in most rats the mammae have reached a secretory condition with thin-walled secrete-filled alveoli. The lobules are large and consist mainly of the numerous distended alveoli; the whole mamma spreads over a considerable area and is thick enough to be palpated in the living animal.

When androgen is injected to female rats after weaning, the mamma undergoes only the initial development which occurs early during the treatment of suckling animals; there sprout acinar buds without secretion. As this effect is already well established by other authors, only few weaned females were studied. In the 2 pubertal animals the collecting ducts remained short but ended in small, thick-walled acini with narrow lumina. In the three 35 day

old rats the mammary anlage consisted of a fairly large mass of undifferentiated epithelium. (Such a stage does not occur during normal development.) The hooded rat treated from the day after weaning on at 37 days possessed small acini which showed both signs of secretion and of fatty involution. Two littermates which had been treated in the same manner showed the same effects on macroscopic inspection, but were not examined histologically.

DISCUSSION

Mammary development is known to require three hormonal factors: estrogen to promote duct growth; progesterone, testosterone, or corticosterone to stimulate budding of acini; and the anterior pituitary lactogenic principle to induce secretion. Whether the sterols exert their influence by way of the pituitary need not be discussed here. However, continued treatment with testosterone during the suckling period as in the experiments reported here leads to full mammary development and secretion. This striking reaction of suckling females to testosterone indicates that one or perhaps both synergistic hormones are supplied by the mother's milk. The observations described above take on some significance in the light of the well-known milk factor of Bittner's in relation to cancer susceptibility.

SUMMARY

While injection of androgen to weaned female young rats promotes the formation of thick-walled mammary alveoli, prolonged treatment of suckling females results in large, lactating glands. It is assumed that the mother's milk may supply other hormones which supplement the effect of the injected testosterone.

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THE EFFECT OF FEEDING THIOURACIL TO HENS UPON THE THYROID GLAND OF CHICKS[1]

RICHTER AND CLISBY (1942), Kennedy (1942), Astwood and co-workers (1943) and MacKenzie and MacKenzie (1943) have shown that a large number of substances related to thiourea inhibit thyroxin secretion, decrease the basal metabolic rate and produce thyroid hypertrophy and hyperplasia in the rat. Of these, 2-thiouracil was the most effective (Astwood 1943). Hughes (1944) produced cretinism by the daily administration of thiouracil to newborn rats and found that the young of thiouracil treated

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[1] Contribution from the Departments of Poultry and Animal Husbandry, Journal Paper No. 232 Purdue University Agricultural Experiment Station.

females showed hyperplasia of the thyroid as early as one day of age. Hughes' results suggest that thiouracil is transmitted by the placenta and mammary gland. Goldsmith et al. (1945) reported that thiourea in the ration of pregnant rats produced thyroid hyperplasia and retarded the growth in the offspring and that the effects of thiourea may be indirectly transmitted through the placenta or through the milk.

Since thiouracil is apparently transmitted by both the rat placenta and mammary gland it seemed probable that it might be transmitted to the egg by the avian genital organs. During the course of a study on the effects of

TABLE 1. THYROID WEIGHTS OF CHICKS FROM THIOURACIL TREATED HENS

Treatment	Date of hatch	No. of chicks	Av. thyroid wt. (mg.)	Per cent of eggs hatched
Controls	5-16-45	9	6.26	57.3
Thiouracil	5-16-45	9	9.56	63.5
Controls	5-29-45	10	6.16	75.4
Thiouracil	5-29-45	10	11.54	61.9
Controls	6-12-45	10	5.26	66.6
Thiouracil	6-12-45	10	10.26	55.2
All Controls		29	5.84	68.1
All Thiouracil		29	10.48	59.7

thiouracil on egg production in the domestic fowl, the thyroid glands of several groups of chicks were examined and evidences of thiouracil effects were found.

METHODS

Two groups of twelve yearling White Leghorn hens each were selected from a group of fowls fed a standard laying ration for a period of several months. On April 1, 1945 thiouracil was added to the ration of one group at a level of 0.2 percent. Three weeks later eggs were selected at random and incubated, and the thyroid glands of three groups of chicks hatched from both the controls and thiouracil-treated females were weighed on a precision balance on the day of hatching. After the first hatch it seemed evident that the thyroid glands of chicks from hens receiving thiouracil were definitely enlarged, and thiouracil analyses of a representative group of eggs were made according to the method of Chesley (1944) as modified by Pashkis et al. (1945).[2]

RESULTS

The thyroid gland weights of three groups of chicks hatched from normal and thiouracil-treated hens are presented in Table 1. Analyses of variance of these data show that in all cases the increases in thyroid weight of the chicks from hens receiving thiouracil are highly significant. Chemical analyses showed that thiouracil was present in the eggs of the treated hens. Egg white contained 0.07–0.15 mg. thiouracil per gram and egg yolk 0.08–0.12 mg. per gram.

There were no observable gross evidences of functional differences in the thiouracil-affected chicks at the time of hatching or during the first week of life. Thiouracil, at the level fed, apparently did not affect egg production,

[2] We are indebted to Mr. R. C. Anderson of the Lilly Research Laboratories for these analyses.

egg size, fertility, or hatchability in adult female fowls. The effects of thiouracil on growth and development of the chicks were not observed because the animals were being used in another experiment involving the susceptibility of the chicks to certain fowl pathogens and death intervened in a high percentage of cases within a short period of time.

SUMMARY

Anatomical and chemical evidence indicates that thiouracil is transmitted through the egg of the domestic fowl to the chick.

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[3] The authors express their appreciation to Dr. K. K. Chen of The Lilly Research Laboratories, for generous supplies of thiouracil and for his counsel during the course of several studies on the effects of thiouracil in the fowl.

NEW BOOK

"Annual Review of Physiology." Vol. VII. By James Murray Luck, Editor. $5.00. +774 pp. Stanford University Press, Stanford University, California, 1945.

The present volume is the seventh in a series of annual reviews of recent literature dealing with, or closely related to, physiology. It contains 26 independent reports each written by an author (or authors) well qualified to summarize and appraise the literature in his or her field. The literature under review extends back from one to four years (usually two), depending generally on the length of time since a given subject has been treated previously in this publication.

There is great need for an up-to-date review of the current literature and it is the purpose of this volume to fulfill that need. The stated policy has been to present "a critical appraisal of recent contributions to the literature in a given field." To that end the success of this volume has been limited, as the editors point out, by the non-availability of many of the foreign journals. From the standpoint of critique, however, it appears that a greater limitation has been imposed by a number of the authors in their failure to give an evaluation of the work under consideration. A further limitation has been the undue amount of side stepping of controversial issues, critical reviews of which would be of great interest. In fairness, it must be pointed out that in a review restricted to recent literature it is often impossible to assess the worth of investigational findings for want of their confirmation or to sketch their perspective in terms of historical background. The selection of only the worthy papers for consideration has constituted a favored method of exercising critical judgment and this has uniformly been done with care. The number of instances found wherein the omission of pertinent data has resulted in an unwarranted interpretation is remarkably few.

Among the excellent contributions in this volume are those by Seifritz, Wright, Schmidt, Kennard, McQuarrie and Barron. These papers reflect superb competence and scholarly enthusiasm. They also demonstrate skillful organization of the literature beforehand, with the result that most citations fall nicely into the line of thought rather than being placed in the topic sentence of paragraph after paragraph in monotonous fashion.

The paper on "Physiological Psychology" is premature. In 28 pages only 70 references are cited and among these the data are often inconclusive. This average of less than 3 citations per page is in contrast to the maximum average for the volume of 14 per page or an average of about 7 per page for the entire volume. It appears that the reader interest is best sustained with an average of from 5 to 8 references per page.

The bare title "Pharmacology" is too all inclusive for an article of limited length and it permits little choice other than collecting a hodge-podge of unrelated data or taking off on a tangent. The present reviewer, who contributed the report under that title for the present volume, adopted the former course as the lesser of two evils. Ingle has given an excellent analysis of se-

lected topics in "Metabolic Functions of the Endocrine System," which is an active and rapidly developing field. R. K. Meyer has done a notable job of condensing the literature in the perpetually voluminous field of "Hormones in Reproduction" covering 314 citations in 24 pages. If the fundamental progress which this body of observations represents seems disproportionately limited, we may properly recall that "Progress in science cannot depend alone upon brilliant inspiration or sudden flight of genius.—In peace and in war progress comes slowly in small new bits, from the unremitting day-by-day labors of thousands of men and women." (President Truman's message to Congress, September 6, 1945.) The *de novo* quality of the endocrine literature suffers from restatement in being reviewed excessively.

In keeping with the shift of emphasis and research effort necessitated by the war, there is included a report on "Applied Physiology" and appropriate cognizance of this type of work is taken elsewhere throughout the volume. Since the practical exploitation of biological data obviously yields quick benefits to mankind, it is an odd commentary that only in time of war is there ordinarily an energetic application of experimental findings.

In a few of the present articles citations to the literature are made by numbers only; in others the names of authors have also been given to a variable extent. The former method of citation, by numbers without names, sacrifices much of the usefulness of the review to those who wish a full acquaintance with the literature.

It is unquestionably of great benefit to investigators to have the voluminous literature on physiological subjects assembled in some readily scannable form. The "Annual Review of Physiology" may serve this purpose to the satisfaction of many. However, in view of the marked variation in quality of the reports and of the fact that an important minority of them have little advantage over a simple abstracting service, the present author feels that serious consideration should continue to be given to the problem of finding an efficient and standardized manner of accomplishing this task. A suggested alternative would be to make available annually a classified bibliography (including titles) with author and subject indices. This would have a number of advantages. (1) It would block the literature into years corresponding with the numerical years and not with an indefinite period. For instance, some of the 1941 literature is reviewed for the first time in the 1945 volume of the "Annual Review of Physiology." (2) It would avoid reduplication of citations, and (3) it could be largely prepared by a staff especially trained for this duty thereby relieving at least 25 skilled investigators annually from a time consuming task. (4) Finally, it would better supplement the comprehensive and critical reviews on physiological subjects which are continually appearing in other publications.

ROY O. GREEP

ASSOCIATION NOTICE

The Government's lifting of the ban on conventions permits the holding of the 1946 scientific assembly of The Association for the Study of Internal Secretions.

Pursuant to the vote of the members at the last business session, this meeting will be held in the same city and on approximately the date of the meeting of the American Medical Association, however, this may be changed at the discretion of the Council. Time and place will be announced later.

Members or others (introduced by members) desiring to present papers should send four copies of an abstract of the work they desire to present to Dr. Carl R. Moore, Department of Zoology, University of Chicago, Chicago 37, Illinois, before April 1, 1946.

ERRATUM

Paper of Hays and Mathieson in August issue, dosage for Epinephrine shown in Tables I and II, on pages 150 and 152 respectively, should read 0.15 instead of 1.5.

THE SQUIBB AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner, in 1941 to Dr. Philip E. Smith, and in 1942 to Dr. Fred C. Koch. A special Committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected due to lack of time. The work cited may be either in the field of pre-clinical or clinical endocrinology. The Award is for $1,200.00. If the recipient should choose to use the Award toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00. The option is left entirely to the recipient.

Each member has the privilege of making one nomination for each Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications, and reprints if possible. Five copies should be sent to the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, not later than March 15, 1946.

E. Kost Shelton, President
Henry H. Turner, Secretary